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(54) Title: MULTIVALENT ANTIGEN–BINDING PROTEINS

(57) Abstract

A multivalent antigen binding protein comprises a single polypeptide chain comprising, in series, two or more single domain binding units which are preferably heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains. Methods for their production and uses thereof, in particular for diagnosis, immunoassay and purification methods are disclosed.

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MULTIVALENT ANTIGEN-BINDING PROTEINS

FIELD OF THE INVENTION

The present invention relates to multivalent and multispecific antigen binding proteins, methods for their production and uses thereof. In particular, the invention relates to antigen binding proteins comprising a polypeptide comprising in series two or more single domain binding units which are preferably variable domains of a heavy chain derived from an immunoglobulin naturally devoid of light chains.

BACKGROUND OF THE INVENTION

Antibodies are protein molecules belonging to a group of immunoglobulins generated by the immune system in response to an antigen. The structure of most antibody molecules is based on a unit comprising four polypeptides, two identical heavy chains and two identical light chains, which are covalently linked together by disulphide bonds. Each of these chains is folded in discrete domains. The C-terminal regions of both heavy and light chains are conserved in sequence and are called the constant regions, comprising one or more so-called C-domains. The N-terminal regions of the heavy and light chains, also known as V-domains, are variable in sequence and determine the specificity of the antibody. The regions in the variable domains of the light and heavy chains (V_L and V_H respectively) responsible for antigen binding activity are known as the hypervariable or complementarity determining regions (CDR).

Natural antibodies generally have at least two identical antigen-binding sites defined by the association of the heavy and light chain variable regions. Individual heavy or light chain domains having the capability to bind antigens have been described in the literature (Ward et al, Nature 341 (1989), 544-546) although generally most naturally occurring antibodies need both a V_H and V_L to form a complete antigen binding site and retain full immunoreactivity.

More recently, immunoglobulins capable of exhibiting the functional properties of the four-chain immunoglobulins described above but which comprise two heavy polypeptide chains and which furthermore are devoid of light polypeptide chains have been described (see European Patent Application EP-A-0584421, Casterman et al, 1994). Methods for the preparation of such antibodies or fragments thereof on a large scale comprising transforming a mould or yeast with an expressible DNA sequence encoding the antibody or fragment are described in patent application WO 94/25591 (Unilever).

The immunoglobulins described in EP-A-0584421, which may be isolated from the serum of Camelids, do not rely upon the association of heavy and light chain variable domains for the formation of the antigen-binding site but instead the heavy polypeptide chains alone naturally form the complete antigen binding site. These immunoglobulins, hereinafter referred to as "heavy-chain immunoglobulins" are thus quite distinct from the heavy chains obtained by the degradation of common (four-chain) immunoglobulins or by direct cloning which contribute part only of the antigen-binding site and

require a light chain partner for antigen-binding, thus forming a complete antigen binding site.

As described in EP-A-0584421, heavy chain immunoglobulin V_H regions isolated from Camelids (forming a complete antigen binding site and thus constituting a single domain binding site) differ from the V_H regions derived from conventional four-chain immunoglobulins in a number of respects, notably in that they have no requirement for special features for facilitating interaction with corresponding light chain domains. Thus, whereas in common (four-chain) immunoglobulins the amino acid residues at the positions involved in the V_H/V_L interaction is highly conserved and generally apolar leucine, in Camelid derived V_H domains this is replaced by a charged amino acid, generally arginine. It is thought that the presence of charged amino acids at this position contributes to increasing the solubility of the camelid derived V_H. A further difference which has been noted is that one of the CDRs of the heavy chain immunoglobulins of EP-A-0584421, the CDR_3, may contain an additional cysteine residue associated with a further additional cysteine residue elsewhere in the variable domain. It has been suggested that the establishment of a disulphide bond between the CDR_3 and the remaining regions of the variable domain could be important in binding antigens and may compensate for the absence of light chains.

In the search for multivalent and multispecific antigen binding proteins, attention has been directed towards the use of fragments, or portions, of a whole antibody which can nevertheless exhibit antigen binding activity. By comparison with the whole antibody, the smaller antibody fragment is

advantageous for use in therapy, for example, as it is likely to be less immunogenic and more able to penetrate tissue.

Binding fragments of common (four-chain) antibodies which have been considered include Fab (light chain associated with the V_H and C_{H1} domains of a heavy chain), F_V (comprising of the V-domains of the heavy and light chains associated with each other) and ScFv (comprising a V_H domain linked to a V_L domain by a flexible peptide linker) fragments. These fragments have only one site for antigen binding compared to the two or more sites contained in the whole antibody, however, and in an attempt to overcome this problem, recombinant fragments having two or more binding sites have been proposed.

In general, those multivalent and/or multispecific constructions which have been described in the literature either comprise two or more polypeptide chains, see for example, patent application WO 94/09131 (Scotgen Limited) and WO 97/14719 (Unilever) or are based on a 'double ScFv' approach, wherein the multivalency arises when two or more monovalent ScFv molecules are linked together, providing a single chain molecule comprising at least four variable domains, as described, for example, in WO 93/11161 (Enzon Inc) and WO 94/13806 (Dow Chemical Co). In all of these cases, the binding site is formed through the association of light and heavy chain variable domains. In WO 93/11161, reference is made to a single-chain protein comprising the binding portions of the variable regions of an antibody light (or heavy) chain but it is stated that as such

proteins are comprised of two similar variable regions, they do not necessarily have any antigen-binding capability.

EP-A-0584421 (Casterman), referred to above, discloses fragments of heavy chain immunoglobulins devoid of light chains, including fragments corresponding to isolated V_H domains or to V_H dimers linked by the hinge disulphide. Further disclosed, but not exemplified, are antibodies having different specificities on each heavy polypeptide chain which could be prepared by combining two heavy chain immunoglobulins or one heavy chain of an immunoglobulin of EP-A-0584421 with a fragment of a conventional four-chain immunoglobulin. There is no suggestion that multivalent and/or multispecific constructs may be prepared by joining together individual V_H domains. Indeed, in the absence of the inherent conformational constraints conferred on the position of the binding sites by the presence of a corresponding light chain, it might generally be expected that the binding domains in constructs of this type would sterically hinder each other, unfavourably influencing binding activity.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides a multivalent antigen binding protein comprising a single polypeptide chain comprising, connected in series, two or more single domain binding units.

In another aspect, the invention provides nucleotide sequences coding for multivalent antigen binding proteins according to the invention and cloning and expression

vectors comprising such nucleotide sequences. Further provided are host cells transformed with vectors comprising such nucleotide sequences and methods of producing antigen binding proteins according to the invention by expression of the nucleotide sequences in such hosts.

The invention also provides compositions comprising multivalent antigen binding proteins according to the invention.

In a further aspect, the invention provides the use of multivalent antigen binding proteins as set forth above in diagnosis or therapy or in other methods for which antibodies or fragments thereof can be used, such as in immunoassay or purification methods. Methods of treatment using the multivalent antigen binding proteins according to the invention are also provided.

In a particular embodiment of the invention, there is provided the use of said multivalent antigen binding proteins in inactivating (bacterio)phages.

By means of the invention, antigen binding proteins having the specificity and binding affinity of the whole immunoglobulin but which have the additional advantage of smaller size are obtained. Furthermore, where the multivalent antigen binding proteins of the present invention comprise variable domains having different antigen specificity, multispecific binding molecules may be obtained. Another advantage is that the constructs according to the invention may conveniently be produced at high yields economically and efficiently on a scale appropriate for industrial use.

The present invention may be more fully understood with reference to the following description, when read together with the accompanying drawings. For convenience, an antigen binding protein according to the invention comprising two single binding units is herein referred to as a 'bihead'.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of *Camelidae* IgG types.

Figure 2 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4638, encoding the heavy chain variable domain of an anti-RR6 antibody (denoted R7) from a llama.

Figure 3 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4640, encoding the heavy chain variable domain of another anti-RR6 antibody (denoted R9) from a llama.

Figure 4 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4601, encoding the heavy chain variable domain of an anti-hCG antibody (denoted H14) from a llama.

Figure 5 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4602, encoding the heavy chain variable domain or another anti-hCG antibody (denoted HI-15) form a llama.

Figure 6 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4603, encoding the heavy chain variable domain of an anti-*Streptococcus* antibody (denoted S36) from a llama.

Figure 7 shows the nucleotide sequence of the *Pst*I-*Bst*EII insert of plasmid pUR4642, encoding the heavy chain variable domain of another anti-*Streptococcus* antibody (denoted S120) from a llama.

Figure 8 shows a map of plasmid pUR4619.

Figure 9 shows the nucleotide sequence within plasmid pUR4619, which encodes an anti-hCG-anti-RR6 bispecific biheaded antigen binding protein (denoted H14-R9), missing the first 4 and last 3 amino acids.

Figure 10 shows the nucleotide sequence within plasmid pUR4620, which encodes an anti-hCG-anti-RR6 bispecific biheaded antigen binding protein (denoted HI15-R7), missing the first 4 and last 3 amino acids.

Figure 11 shows the nucleotide sequence within plasmid pUR4621, which encodes an anti-hCG-anti-RR6 bispecific biheaded antigen binding protein (denoted HI15-R9), missing the first 4 and last 3 amino acids.

Figure 12 shows the nucleotide sequence within plasmid pUR4622, which encodes a homodimeric bivalent anti-RR6 antigen binding protein (denoted R7-R7), missing the first 4 and last 3 amino acids.

Figure 13 shows the nucleotide sequence within plasmid pUR4623, which encodes a heterodimeric bivalent anti-RR6 antigen binding protein (denoted R7-R9).

Figure 14 shows the results of an hCG/RR-6 bispecific binding assay.

Figure 15 shows the results of a RR6/RR6 bifunctional binding assay.

Figure 16 shows the binding activity and SDS-PAGE analysis of crude *P. Pastoris* supernatants expressing the constructs of Example 4.

Figure 17 shows a plasmid map of pHP14.3A.

Figure 18 shows the binding activity and SDS-PAGE analysis of crude *H. polymorpha* supernatants expressing the constructs of Example 5.

Figure 19 shows the reduction of infectivity of lactic acid bacteria phages using the constructs according to the invention.

Figure 20 shows a diagrammatic representation of the use of a biheaded antibody to form an active binding layer.

Figure 21 shows the capture of I^{125} labelled hCG to antibody adsorbed and double headed antibody fragment sensitised RR6-BSA wells.

Figure 22 shows a schematic representation of the use of a bispecific biheaded antibody fragment of the invention in an immunoassay to detect hCG antigen.

Figure 23 shows the assay response of latex made by adsorption of a monoclonal antibody and self-assembled bihead/RR6-BSA latex.

Figure 24 shows the assembly of a bispecific antibody fragment on a dextran surface coated with RR6-BSA.

Figure 25 shows acidification curves of milk by the lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* LM0230 with and without phage P2 and/or bihead 3850 (also referred to as bihead 3-2).

Figure 26 shows the A405 signals of an ELISA to determine bispecificity of H14-R9 biheads A405.

Figure 27 shows the nucleotide sequence within plasmid pUR4618 which encodes an anti-hcg anti-RR6 bispecific biheaded antigen binding protein (denoted H14-R7, missing the first 4 and last 3 amino acids.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the finding that the variable domains of a heavy chain derived from an immunoglobulin naturally devoid of light chains may be joined together to form a multivalent single polypeptide which retains the antigen binding affinity of the parent whole immunoglobulin but which is much smaller in size and therefore less immunogenic, thereby providing important benefits over the use of whole antibody molecules, particularly, for example, in the area of diagnostics, therapy and targeting. Accordingly, the invention as described herein is directed to multivalent forms of antigen binding proteins, methods for preparing them and new and improved methods for their use.

As used herein, a multivalent antigen binding protein is a protein which has more than one antigen binding site.

Included within this are bivalent, trivalent, tetravalent and so on. According to one aspect, bivalent forms, that is forms having two antigen-binding sites, are preferred but it will be appreciated that higher multivalent forms may find application in certain circumstances.

A single domain binding unit means an immunoglobulin variable domain which naturally forms a complete antigen binding site.

The single domain binding units for use according to the present invention are preferably heavy chain variable domains derived from any immunoglobulin naturally devoid of

light chains, such that the antigen-binding site is located exclusively in the heavy chain variable domain. Preferably, the heavy chain variable domains for use in the invention are derived from immunoglobulins naturally devoid of light chains such as may be obtained from camelids as described in EP-A-0584421, discussed above.

Where the individual single domain binding units which are joined together to form the multivalent antigen binding proteins of the invention have the same antigen specificity, a binding protein which binds more than one molecule of the same type will be produced. Alternatively, multivalent and multispecific binding proteins according to the invention which are able to bind different epitopes from each other may be obtained by assembling together single domain binding units directed against different antigens. Heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains having a determined antigen specificity may conveniently be obtained by screening expression libraries of cloned fragments of genes encoding camelid immunoglobulins generated using conventional techniques, as described, for example, in EP-A-0584421 and Example 1.

The multivalent antigen-binding proteins of the invention may be formed by linking together the single domain binding units in series, such that each single domain binding unit is linked to at least one other variable domain.

The individual single domain binding unit may be linked sequentially by means of peptide linkers, conveniently flexible peptide linkers which allow the domains to flex in

relation to each other such that simultaneous binding to multiple antigenic determinants may be achieved. It will be appreciated that the binding of the linker to the individual single domain binding unit will be such that it does not affect the binding capacity of the single domain antigen binding site. Any peptide linker which permits the single domain binding units components to be linked in such a way that each variable domain retains the binding specificity of the whole immunoglobulin from which it is derived may suitably be used. Such linkers include, e.g., peptides derived from known proteins, such as glucoamylase, cellobiohydrolase, or cell wall proteins (CWP), or synthetic peptides which are rationally designed. The linker may suitably comprise from 1 to 400 or more amino acid residues; conveniently, the peptide linker comprises from 5 to 20 amino acid residues. This group of antigen binding proteins according to the invention with such a linker between the two single domain binding units is usually preferred because of its good production yields.

In another preferred embodiment of the invention, the individual single domain binding units may be connected directly in series without any intervening linker. In this way, the binding sites in the multivalent binding proteins according to the invention are held in much closer proximity to each other than would be the case in the whole immunoglobulin from which the immunoglobulin fragments are derived. It might generally be expected that this would give rise to unfavourable steric interactions, but surprisingly, full binding activity is found to be retained. Furthermore, these fragments with directly linked single domain binding

units appear to be more stable, e.g. towards proteolytic degradation.

In an alternative embodiment, functional groups such as enzymes may be fused to the antigen binding protein.

The multivalent antigen binding proteins according to the invention may suitably find application in a wide variety of uses for which antibodies, or fragments thereof, have been proposed in the art. These uses include diagnosis, therapy, targeting, immunoassays, in agglutination, agglutination assays and purification processes, and detergents.

For use in diagnosis or therapy targeting, antigen binding proteins according to the invention having binding activity directed against both target site and the diagnostic or therapeutic agent may be constructed. Multivalent binding proteins having two or more distinct binding specificities are of particular use, for example, in the targeted delivery of therapeutic agents to their intended site of action. The binding proteins according to the invention establish the connection between therapeutic agent and target site by self-assembly, thereby avoiding the need for chemical conjugation reactions, and the therapeutic agent is guided to the target, giving increased local efficiency. Cytotoxic agents may be targeted directly to the tumour cell to be attacked, for example, by means of a bispecific, bivalent binding protein according to the invention having specificity for both the cell and the cytotoxic agent. Enzymes which are capable of generating a cytotoxic product at a target site, particularly oxido-reductases such as glucose oxidase (which catalyses the oxidation of glucose to

gluconic acid, thereby producing hydrogen peroxide which exhibits cell toxicity) similarly can conveniently be delivered to the intended site of action using a binding protein according to the invention having both anti-target and anti-enzyme specificity.

Alternatively, the antigen binding proteins according to the invention may conveniently be attached to one or more appropriate diagnostically or therapeutically effective agents or carriers by methods conventional in the art. Direct attachment of the diagnostically or therapeutically effective agent to the small antigen binding proteins of the invention runs the risk that the binding activity will be adversely affected through steric hindrance of the binding site. In a particular embodiment, therefore, the antigen binding protein has an additional polypeptide group appended to it, which additional polypeptide group does not contribute to the binding properties but which provides a "handle" for the attachment of the diagnostic or therapeutic agent.

Such antigen binding proteins with an attached polypeptide also find particular application in immunoadsorption processes, especially immunoaffinity purification processes which require that the binding protein be linked to another material, for example a label, such as an enzyme, or a solid phase, for example, a carrier material in a column.

The additional peptide group is generally attached to the antigen binding protein at or near one end of its polypeptide chain through a peptide bond, such that this polypeptide chain is prolonged by the additional peptide

which now forms the terminal portion of the chain. Conveniently, the additional peptide group will be attached at its amino terminus. Suitable additional peptide linking groups and methods for their attachment are described in WO 91/08492 (Unilever). Conveniently, they comprise at least 5 but preferably not more than 20 amino acid residues and preferably include at least one lysine residue as this provides a convenient site for covalent attachment onto surfaces or proteinaceous tracers such as enzymes. A particularly suitable additional peptide linking group, particularly for coupling the antigen binding protein to a solid plastics surface, comprises the "Myc" amino acid sequence:

GLU-GLN-LYS-LEU-ILE-SER-GLU-GLU-ASP-LEU-ASN.

(see SEQ ID. NO: 1)

Coupling of the additional peptide to a solid surface such as latex particles and other structures formed from plastics material commonly used in immunoassays may conveniently be achieved by means of conventional chemical cross-linking agents. It will be appreciated that the chemical coupling site in the additional peptide should preferably be sufficiently remote from the variable domain binding sites such that the coupled molecule does not affect binding activity. Alternatively, therapeutic agents and tracers such as enzymes (for example horse radish peroxidase, alkaline phosphatase, glucose oxidase) may be covalently coupled to the additional peptide via the E-amino (epsilon)group of the lysine group.

Multivalent, multispecific binding proteins according to the invention may be used to particular advantage in adsorption and purification techniques by value of their ability to exhibit specificity for two or more distinct materials. The detection and purification of ligands may conveniently be achieved using surfaces activated with constructs according to the invention. By way of illustration, a suitable support incorporating molecules for which a binding protein according to the invention has binding specificity can be activated or sensitised by coating it with a bispecific binding protein of appropriate binding specificity, the remaining binding specificity being free to bind with analyte or contaminant as appropriate depending on the intended use. Suitable molecules which can be incorporated into the support, either by adsorption or covalent bonding, include proteins, peptides, carbohydrates, DNA, RNA or conjugates thereof. Particularly preferred ligands which may be detected or purified in this way include human chorionic gonadotrophin, luteinising hormone, estrone, progesterone or metabolites thereof. The support may be particulate, planar or porous in nature. Suitable supports include those conventionally used in immuno-adsorption and purification techniques, particularly latex particles, polystyrene wells and dextran surfaces.

In an alternative aspect, binding proteins according to the invention may be used as cross-linking reagents. For example, a bispecific binding protein can link one phage to another, thereby leading to their inactivation. Inactivation of viruses or microorganisms may similarly be accomplished through agglutination.

In a further embodiment of the invention, binding proteins may be used in detergent compositions, and the like, for the treatment of stains essentially as described in PCT/EP 98/03438. Thus, for example, a bispecific protein according to the invention can have high binding affinity for stain as one specificity and for enzyme as another one. Such a bispecific protein could fulfil the requirement of accumulating enzyme on stain either by supplying said protein together with enzyme as a pre-formed non-covalent complex or by supplying the two separately and allowing them to self-assemble either in the wash liquor or on the stain. A further important aspect is to use a binding protein that binds to several different, but structurally-related, molecules in a class of "stain substances". This would have the advantage of enabling a single enzyme species to bind (and bleach) several different stains. An example would be to use a binding protein which binds to the polyphenols in wine, tea, and blackberry.

Multivalent antigen binding proteins according to the invention may be prepared by transforming a host by incorporating a gene encoding the polypeptide as set forth above and expressing said gene in said host.

Suitably the host or hosts may be selected from prokaryotic bacteria, such as Gram-negative bacteria, for example *E. coli*, and Gram-positive bacteria, for example *B. subtilis* or lactic acid bacteria, lower eukaryotes such as yeasts, for example belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*, or moulds such as those belonging to the genera *Aspergillus* or *Trichoderma*.

Preferred hosts for use in connection with the present invention are the lower eukaryotic moulds and yeasts.

Techniques for synthesising genes, incorporating them into hosts and expressing genes in hosts are well known in the art and the skilled person would readily be able to put the invention into effect using common general knowledge.

Methods for producing antibody fragments or functionalised fragments thereof derived from the heavy chain immunoglobulin of *Camelidae* using a transformed lower eukaryotic host are described, for example in patent application WO 94/25591 and such techniques may suitably be applied to prepare constructs according to the present invention.

Proteins according to the invention may be recovered and purified using conventional techniques such as affinity chromatography, ion exchange chromatography or gel filtration chromatography.

The activity of the multivalent binding proteins according to the invention may conveniently be measured by standard techniques known in the art such as enzyme-linked immunoadsorbant assay (ELISA), radioimmune assay (RIA) or by using biosensors.

The following examples are provided by way of illustration only. Techniques used for the manipulation and analysis of nucleic acid materials were performed as described in Sambrook et al, Molecular Cloning, Cold Spring Harbor Press, New York, 2nd Ed.(1989) unless otherwise indicated.

HC-V denotes heavy chain variable domain.

Restriction sites are underlined.

EXAMPLES

EXAMPLE 1. Induction of humeral immune responses in llama.

Male llamas were immunised with a water in oil emulsion (1:9 V/V, antigen in water: Specol (Bokhout et al.) subcutaneously and intramuscularly. Per immunisation site 0.75-1.5 ml water in oil emulsion was inoculated containing 100:g antigen. The antigens used were: hCG (Sigma), azo-dye RR6 (ICI) which was coupled to BSA via its reactive triazine group and *Streptococcus mutans* HG982 cells. Immunisations were performed according to the following time table: The second immunisation was performed three weeks after the first. The third was performed two weeks after the second immunisation. The immune response was followed by antigen specific ELISAs.

The anti-RR-6 response was measured by using Nunc Covalink plates, which where coated with the azo-dye. After incubation with (diluted) serum samples, the bound llama antibodies were detected via a incubation with poly-clonal rabbit-anti-llama antiserum (obtained via immunising rabbits with llama immunoglobulines which were purified via ProtA and ProtG columns; ID-DLO), followed by an incubation with swine-anti-rabbit immunoglobulines (Dako) conjugated with alkaline phosphatase. Finally the alkaline phosphatase enzyme-activity was determined after incubation with p-nitro-phenyl phosphate and the optical density was measured at 405nm. The anti-hCG response, was measured in essentially the same way using Nunc maxi-sorb plates coated with hCG. The anti-*Streptococcus* response, was measured in essentially

the same way using nunc maxi-sorb plates sensitised with *Streptococcus mutans* HG982.

EXAMPLE 2. Cloning, expressing and screening of llama HC-V fragments.

2.1 Isolation of gene fragments encoding llama HC-V domains.

From an immunised llama a blood sample of about 200ml was taken and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynnski and Sacchi, 1987). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding HC-V fragments and part of the long or short hinge region were amplified by PCR using specific primers:

*Pst*I

V_H - 2B 5'-AGGTSMARCTGCAGSAGTCWGG-3' (see SEQ. ID. NO: 2)

S = C and G, M = A and C, R = A and G, W = A and T,

*Hin*dIII

Lam-07 5'-AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTG GTGCG-3'

(short hinge) (see SEQ. ID. NO: 3)

*Hin*dIII

Lam-08 5'-AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTT GGGTT-3'

(long hinge) (see SEQ. ID. NO: 4)

Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the HC-V domain, encoding the amino acids L-Q) and *Bst*EII (located at the 3'-end of the HC-V gene fragments, coinciding with the amino acid sequence Q-V-T), the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel.

2.2 Construction of *Saccharomyces cerevisiae* expression plasmids encoding llama HC-V domains.

Plasmids pUR4547 and pUR4548 are *Saccharomyces cerevisiae* episomal expression plasmids, derived from pSY1 (Harmsen et al., 1993). From pSY1 the *Pst*I site, located in front of the GAL7 promoter was removed after partial digestion with *Pst*I, incubation with Klenow fragment and subsequent blunt end ligation. After transformation the desired plasmid could be selected on the basis of restriction pattern analysis. Subsequently, the *Bst*EII site in the Leu2 selection marker was removed by replacing the about 410bp *Afl*II/*Pfl*MI fragment with a corresponding fragment in which the *Bst*EII site was removed via a three step PCR mutagenesis, using the primers:

PCR-A:

*Pfl*MI

BOLI 1 5'-GGGAATTCCAATAGGTGGTTAGCAATCG

(see SEQ. ID. NO: 5)

(*Bst*EII)

BOLI 4 5'-GACCAACGTGGTCGCCTGGCAAAACG

(see SEQ. ID. NO: 6)

PCR-B:

(*Bst*EII)

BOLI 3 5'-CGTTTTGCCAGGCGACCACGTTGGTC (see SEQ. ID. NO: 7)

*Afl*II

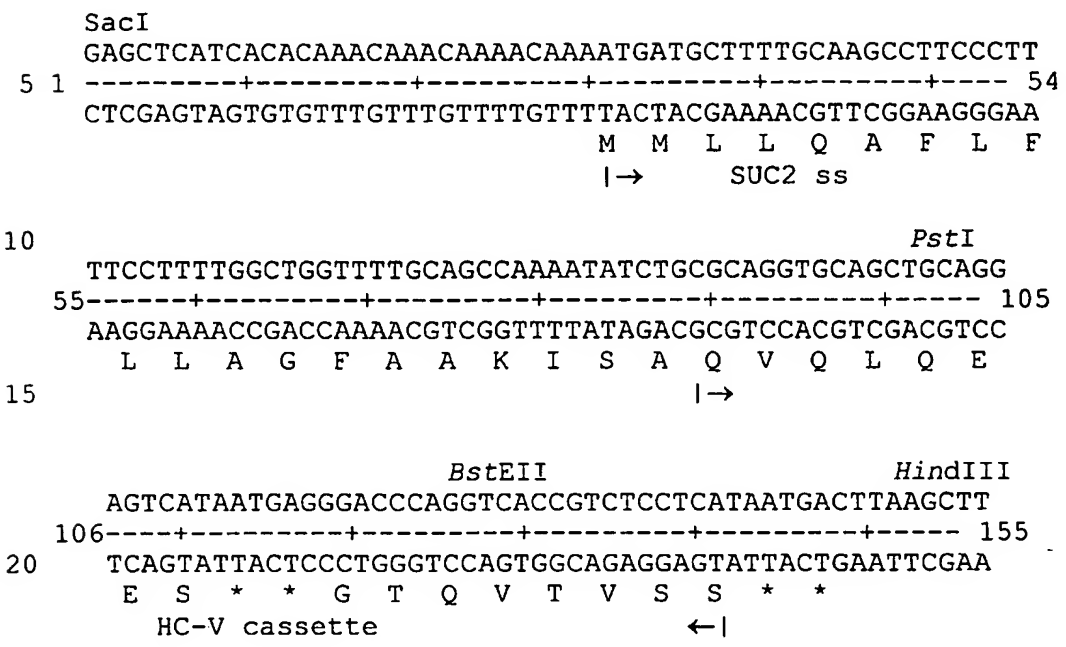
BOLI 2 5'-CCCCAAGCTTACATGGTCTTAAGTTGGCGT

(see SEQ. ID. NO: 8)

PCR-A was performed with primers BOLI 1 and BOLI 4 and resulted in an about 130bp fragment with the *Pfl*MI restriction site at the 3'-end and the inactivated *Bst*EII site at the 5'-end. PCR-B was performed with primers BOLI 2 and BOLI 3 and resulted in an about 290bp fragment with the *Afl*II site at the 5'-end. The third PCR was with the fragments obtained from reaction A and B, together with the primers BOLI 1 and BOLI 2.

Finally, the about 1.8kb *Sac*I-*Hin*dIII fragment was replaced with synthetic fragments, having sequences as presented below, resulting the plasmids pUR4547 and pUR4548, respectively.

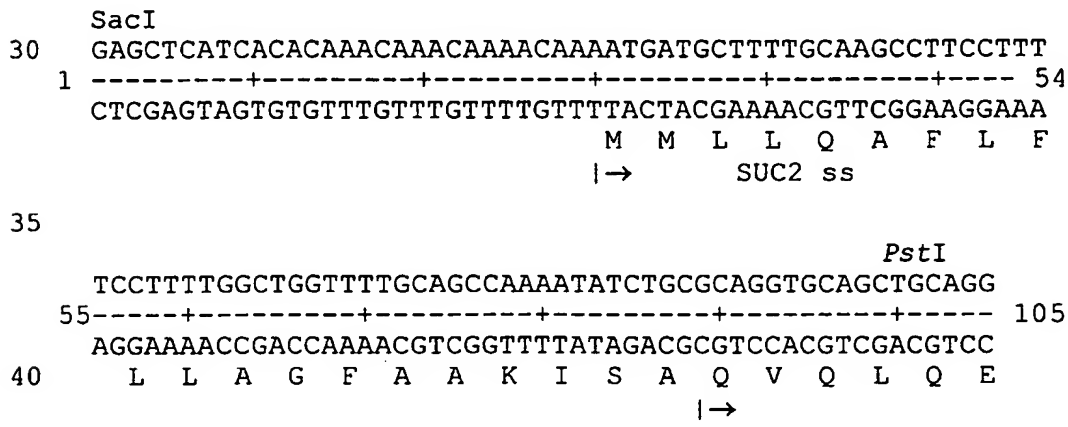
- *Sac*I/*Hin*dIII fragment of pUR4547

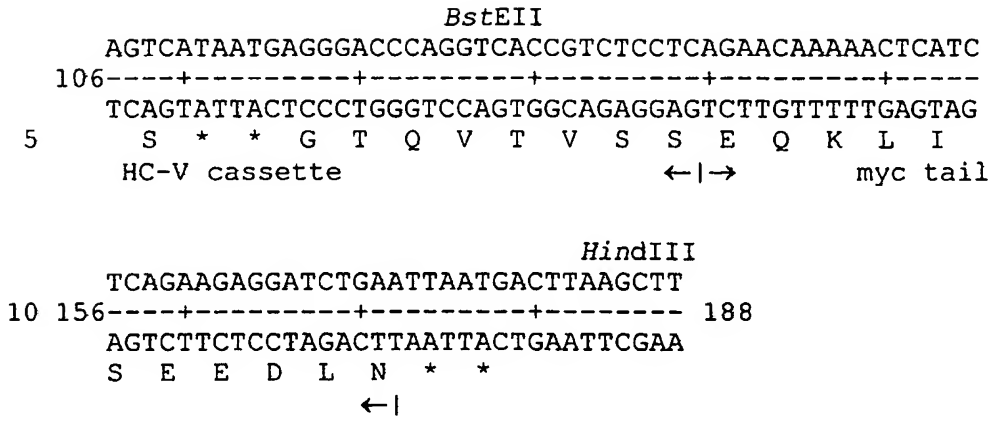


(see SEQ. ID. NO: 9 and NO: 10)

and

- *Sac*I/*Hin*dIII fragment of pUR4548





(see SEQ. ID. NO: 11 and NO: 12)

Both plasmids contain the GAL7 promoter and PGK terminator sequences as well as the invertase (SUC2) signal sequence. In both plasmids the DNA sequence encoding the SUC2 signal sequence is followed by the first 5 codons, (encoding Q-V-Q-L-Q = SEQ. ID. NO: 13) of the HC-V domain (including the *Bst*II site), a stuffer sequence, the last six codons (encoding Q-V-T-V-S-S = SEQ. ID. NO: 14) of the HC-V domain. In pUR4547, this is followed by two stop codons, an *Afl*II and *Hin*dIII site. In pUR4548, this sequence is followed by eleven codons encoding the myc-tag, two stop codons, an *Afl*II and *Hin*dIII site.

Plasmids pUR4547 and pUR4548 were deposited under the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Baarn on 18th August 1997 with deposition numbers: CBS 100012 and CBS 100013, respectively. In accordance with Rule 28(4) EPC, or a similar arrangement from a state not being a contracting state of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an expert only.

Upon digesting pUR4548 with *Pst*I and *Bst*EII, the about 6.4kb vector fragment was isolated and ligated with the *Pst*I-*Bst*EII fragments of about 350bp obtained as described above. After transformation of *S. cerevisiae*, via electroporation, transformants were selected from minimal medium agar plates (comprising 0.7% yeast nitrogen base, 2% glucose and 2% agar, supplemented with the essential amino acids and bases).

2.3 Screening for antigen specific HC-V domains.

For the production of llama HC-V fragments with myc-tail, individual transformants were grown overnight in selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and subsequently diluted ten times in YPGal medium (comprising 1% yeast extract, 2% bacto pepton and 5% galactose). After 24 and 48 hours of growth, the culture supernatant of the colonies was analysed by ELISA for the presence of HC-V fragments which specifically bind to the antigens hCG, RR6 or *Streptococcus* in essential the same way as described in Example 1. In this case, however, the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase. In this way a number of anti-hCG, anti-*Streptococcus* and anti-RR6 HC-V fragments have been isolated, among which are:

anti-RR6:

R7	pUR4638	(see Figure 2; SEQ. ID. NO: 15 and NO: 16)
R9	pUR4640	(see Figure 3; SEQ. ID. NO: 17 and NO: 18)

anti-hCG (alpha unit):

H14 pUR4601 (see Figure 4; SEQ. ID. NO: 19 and NO: 20)

HI15 pUR4602 (see Figure 5; SEQ. ID. NO: 21 and NO: 22)

anti-*Streptococcus*:

S36 pUR4603 (see Figure 6; SEQ. ID. NO: 23 and NO: 24)

S120 pUR4642 (see Figure 7; SEQ. ID. NO: 25 and NO: 26)

EXAMPLE 3. Production of llama HC-V biheads by *S. cerevisiae*.

3.1 Construction of episomal expression plasmids encoding anti-hCG/anti-RR6 bispecific biheads.

In the anti-hCG HC-V fragments H14 and HI15 (anti-alpha-subunit), the *Pst*I site was removed and a *Xho*I site was introduced via PCR, using the primers:

MPG158WB

*Xho*I

5'-GAATTAAGCGGCCGCCCAGGTGAAACTGCTCGAGTCWGGGGGA-3'

(see SEQ. ID. NO: 27)

and

MPG159WB

*Bst*EII

3'-CCCTGGGTCCAGTGGCAGAGGAGTGGCAGAGGAGTCTTGTTT-5'

(see SEQ. ID. NO: 28)

In this way the sequence:

*Pst*I

CAG GTC CAG CTG CAG GAG TCT GGG

Q V Q L Q E S G

(see SEQ. ID. NO: 29 and NO: 30)

became

```
                    XhoI
CAG GTG AAA CTG CTC GAG TCW GGG
 Q   V   K   L   L   E   S   G
```

(see SEQ. ID. NO: 31 and NO: 32)

Upon digesting the PCR fragments with *Xho*I and *Bst*EII, the about 330bp fragments were purified via agarose gel electrophoresis and isolation from the gel. The fragments were cloned into pUR4421 (see Example 1 in WO 94/25591) which was digested with the same enzymes, resulting in pJS2 (H14) and pJS3 (HI15). Subsequently, the about 420bp *Eag*I - *Hin*dIII fragments of pJS2 and pJS3 were isolated and ligated in the about 6.6kb *Eag*I- *Hin*dIII vector fragment of the pSY1 plasmid of which the *Pst*I and *Bst*EII sites were removed as described in Example 2.2. The resulting plasmids pJS7 and pJS8, respectively, were digested with *Bst*EII and *Hin*dIII, after which the purified vector fragment was religated in the presence of a synthetic linker having the following sequence:

```
BstEII                    PstI                    HindIII
 <-              MPG 160 WB (49)                  ->
GGTCACCGTCTCCTCACAGGTGCAGCTGCAGGAGTCACTGTAATGACTTAAGCTT
---------+---------+---------+---------+---------+----- 55
CCAGTGGCAGAGGAGTGTCCACGTCGACGTCCTCAGTGACATTACTGAATTCGAA
      <-          MPG 161 WB (48)                    ->
 V  T  V  S  S  Q  V  Q  L  Q  E  S  L  *  *  L  K  L
```

MPG 160 WB (49) (see SEQ. ID. NO: 33)

MPG 161 WB (48) (see SEQ. ID. NO: 34)

resulting in plasmids pJS9 and pJS10. Finally, these plasmids were digested with *Pst*I and *Hin*dIII, after which the purified vector fragments of about 7.0kb were ligated

with the *Pst*I -*Hin*dIII fragments of about 350bp of pUR4638 and pUR4640, encoding the anti-RR6 HC-V fragments R7 and R9, respectively, followed by the myc-tail. The resulting *S. cerevisiae* episomal expression plasmids pUR4618, pUR4619, pUR4620 and pUR4621 encode a anti-hCG-anti-RR6 bispecific bihead preceded by the SUC2 signal sequence and followed by the myc-tail.

pUR4618: SUC2 - H14 - R7 - myc (see Figure 27; SEQ. ID. NO: 35 and NO: 36)

pUR4619: SUC2 - H14 - R9 - myc (see Figures 8-9; SEQ. ID. NO: 37 and NO: 38)

pUR4620: SUC2 - HI15 - R7 - myc (see Figure 10; SEQ. ID. NO: 39 and NO: 40)

pUR4621: SUC2 - HI15 - R9 - myc (see Figure 11; SEQ. ID. NO: 41 and NO: 42)

Upon digesting these plasmids with *Xho*I and partially with *Bst*EII, *Xho*I-*Bst*EII fragments of about 0.7kb can be isolated and subsequently cloned into the vector fragment of pUR4547 (digested with the same enzymes). In this way biheads can be obtained without the myc tail.

It will be appreciated that expression vectors can be constructed in which different promoter systems, e.g. the constitutive GAPDH promoter or different signal sequences, e.g. the mating factor prepro sequence are used.

3.2 Construction of episomal expression plasmids encoding anti-RR6 bivalent biheads.

Upon digesting plasmids pUR4618 and pUR4619 with *Bst*EII (partially) and *Hin*dIII, DNA fragments of about ~440 and ~400 bp could be purified, respectively. These fragments were subsequently ligated with the *Bst*EII-*Hin*dIII vector fragment (~6.7 kb) of pUR4638 resulting in pUR4622 and pUR4623 (see Figures 12-13; SEQ. ID. NOS: 43/44 and 45/46, respectively), encoding a homodimeric bivalent and a heterodimeric bivalent bihead, respectively.

pUR4622: SUC2 - R7 - R7 - myc
pUR4623: SUC2 - R7 - R9 - myc

3.3 Production and analysis of the HC-V biheads.

After introducing the expression plasmids pUR4618 through pUR4623 into *S. cerevisiae* via electroporation, transformants were selected from minimal medium agar plates as described in Example 2.3. For the production of biheads, the transformants were grown overnight in selective minimal medium and subsequently diluted ten times in YPGal medium. After 24 and 48 hours of growth, samples were taken for Western blot analysis. For the immuno detection of the produced biheads via Western blot analysis, monoclonal anti-myc antibodies were used, followed by incubation with polyclonal rabbit-anti-mouse conjugate with alkaline phosphatase.

Bi-functionality of the bispecific biheads was tested as follows:

PINs coated with hCG were incubated with (diluted) medium samples. Subsequently, the PINs were incubated with a RR6-alkaline phosphatase conjugate, in which the azo-dye RR6 was coupled to the alkaline phospathase via its reactive triazine group. Finally the alkaline phosphatase enzyme-activity was determined after incubation of the PINs with p-nitro-phenyl phosphate and the optical density was measured at 405nm (see Figure 14).

Bi-functionality of the mono-specific, bivalent biheads was tested as follows:

Nunc Covalink plates, coated with RR6 were incubated with (diluted) medium samples. Subsequently, they were incubated with a RR6-alkaline phosphatase conjugate, in which the azo-dye RR6 was coupled to the alkaline phospathase via its reactive triazine group. Finally the alkaline phosphatase enzyme-activity was determined after incubation with p-nitro-phenyl phosphate and the optical density was measured at 405nm (see Figure 15).

EXAMPLE 4. Production of HC-V biheads by *P. pastoris*.

4.1 Construction of integration vectors for the expression of anti-hCG/anti-RR6 bispecific biheads.

To allow the expression and secretion of the Llama bihead constructs in *P. pastoris*, the gene encoding the bispecific construct was fused to the alpha-mating factor leader sequence in the commercially available *P. pastoris* expression vector pPIC9 (Invitrogen). The construction of the final expression vectors involved several cloning steps.

Step 1: The construction of the bispecific HCV expression vectors required the construction of two shuttle vectors, pPIC9N and pUC.HCVx2. For pUC.HCVx2 the *Hin*dIII/EcoRI polylinker of pUC19 was replaced with a synthetic *Hin*dIII/EcoRI fragment, destroying the original *Hin*dIII site, introducing a NheI site which allows the direct fusion to the alpha-Mating Factor leader sequence in pPIC9N, and introducing the XhoI and *Hin*dIII HCVx2 insertion sites.

Synthetic insert of pUC.HCVx2:

```
  ----A  S  Q  V  K  L  L  E-----
AAGCTGCTAGCCAGGTGAAACTGCTCGAGCCCGGGAAGCTTGAATTC
     NheI              XhoI      HindIII
```

(see SEQ. ID. NO: 47 and NO: 48)

The synthetic linker was constructed by annealing the synthetic oligonucleotides PCR.650 and PCR.651.

PCR.650: 5'-AGCTGCTAGCCAGGTGAAACTGCTCGAGCCCGGGAAGCTTG-3'

(see SEQ. ID. NO: 49)

PCR.651: 5'-AATTCAAGCTTCCCGGGCTCGAGCAGTTTCACCTGGCTAGC-3'

(see SEQ. ID. NO: 50)

The XhoI/*Hin*dIII gene fragments encoding the bispecific HCV fragments were excised from pUR4619 and pUR4621 (see Example 3.1) and inserted into the XhoI/*Hin*dIII opened pUC.HCVx2 shuttle vector, thus yielding the intermediate constructs pUC.HCV.19 and pUC.HCV21. For pPIC9N the XhoI/EcoRI polylinker of pPIC9 (Invitrogen) was replaced with a synthetic XhoI/EcoRI fragment which introduces a NheI

restriction site immediately downstream of the alpha-Mating Factor leader sequence.

Synthetic insert of pPIC9N:

L E K R A S-----
CTCGAGAAAAGAGCTAGCCCCGGGGAATTC
XhoI NheI EcoRI

(see SEQ. ID. NO: 51 and NO: 52)

The new insert was constructed by annealing the synthetic oligonucleotides PCR.648 and PCR.649.

PCR.648: 5'-TCGAGAAAAGAGCTAGCCCCGGGG-3'

(see SEQ. ID. NO: 53)

PCR.649: 5'-AATTCCCCGGGGCTAGCTCTTTTC-3'

(see SEQ. ID. NO: 54)

Step 2: The final expression vectors were constructed via a three point ligation. The BamHI/NheI fragment from pPIC9N which contains the alpha-Mating Factor encoding sequence and the NheI/EcoRI HCVx2 inserts from pUC.HCV21 and pUC.HCV19 were cloned together into a BamHI/EcoRI opened pPIC9 vector. This resulted in the isolation of the *P. pastoris* transformation and expression vectors pPIC.HCV19 and pPIC.HCV21 respectively.

4.2 Production and analysis of HC-V biheads.

Step 1: Transformation and selection of transformed *P. pastoris* cells:

P. pastoris cells were transformed essentially as described by Faber et al. Briefly: *P. pastoris* GS115 cells were grown overnight at 30EC in 500ml YPD medium (1% Yeast Extract, 2% Peptone, 1% Glucose) to OD_{600}=1.4. The cells were spun and the pellet was washed with sterile distilled water before re-suspending in 100ml KDTT buffer (50mM Potassium Phosphate pH 7.5, 25mM DTT). After 15 minutes incubation at 37EC the cells were pelleted (3 minuntes 3000 rpm) and re-suspended in 100ml ice-cold STM buffer (92.4g Glucose/l, 10mM Tris.HCl pH 7.5, 1mM $MgCl_2$). After 5 washes with this buffer the cell pellet was re-suspended in a final volume of 0.5ml STM buffer. Approximately 2-5 µg DNA in 2 µl H_2O (BglII digested pPIC constructs: DNA purified via Phenol/Chloroform extractions and precipitation) was mixed with 70 µl of fresh competent *P. pastoris* cells (on ice). The cells were electroporated in a 0.2cm cuvette at 1.5 kV, 400, 25 µF in a BioRad Gene-Pulser. Immediately after electroporation, 1ml of YPD medium was added to the cells. After recovery for 1 hour at 30EC, the cells were pelleted and re-suspended in 200 µl 1M Sorbitol and plated out onto MD plates (1.34% YNB, $4x10^{-5}$% Biotin, 1% Glucose, 0.15% Agar). Colonies formed by transformed cells (His^+) were visible within 48 hours incubation at 30EC. Transformed *P. pastoris* cells GS115 were selected essentially as recommended by the Invitrogen Pichia pastoris expression manual. The plates containing the His^+ transformants were used to screen for the Mut^+ and Mut^S phenotype as follows: Using sterile toothpicks, colonies were patched on both an MM plate (1.34% YNB, $4x10^{-5}$% Biotin, 0.5% MeOH, 0.15% Agar) and an MD plate, in a regular pattern, making sure to patch the MM plate first. Approximately 100 transformants were picked for each construct. After incubating the plates at 30EC for 2-3 days

the plates were scored. Colonies that grow normally on the MD plates but show little or no growth on the MM plates were classified as Mut^S clones.

Step 2: Production and evaluation of the bispecific HC-V biheads.

Transformed and selected *P. pastoris* clones were induced to express bispecific antibody using the protocol outlined below:

1) Using a single colony from the MD plate, inoculate 10ml of BMGY (1% Yeast Extract, 2% Peptone, 100mM potassium phosphate pH6.0, 1.34% YNB, $4x10^{-5}$% Biotin, 1% Glycerol) in a 50ml Falcon tube.
2) Grow at 30EC in a shaking incubator (250 rpm) until the culture reaches an OD_{600}=2-8.
3) Spin the cultures at 2000g for 5 minutes and re-suspend the cells in 2ml of BMMY medium (1% Yeast Extract, 2% Peptone, 100mM potassium phosphate pH6.0, 1.34% YNB, $4x10^{-5}$% Biotin, 0.5% Glycerol).
4) Return the cultures to the incubator.
5) Add 20 µl of MeOH to the cultures after 24 hours to maintain induction.
6) After 48 hours harvest the supernatant by removing the cells by centrifugation.

The crude supernatants were tested for the presence of HC-V bihead fragment via analysis on 12% acrylamide gels using the Bio-Rad mini-Protean II system (Figure 16). Bispecific binding activity via shown via ELISA as follows:

1) 96 well ELISA plates (Greiner HC plates) were activated overnight at 37EC with 200 µl/well of the BSA-RR6 conjugate (see Example 1) in PBS.
2) Following one wash with PBST the wells were incubated for 1 hour at 37EC with 200 µl blocking buffer per well. *Blocking buffer*: 1% BSA in PBS-T.
3) Serial dilutions of test samples (100 µl) were mixed with equal volumes of blocking buffer and added to the sensitised ELISA wells. Incubated at 37EC for 1-2 hours.
4) 200 µl hCG-AP conjugate in blocking buffer was added to each well in which the hCG was coupled to the alkaline phospathase via glutaraldehyde coupling.
5) Following one wash with PBST captured hCG-AP was detected by adding 100 µl/well pNPP substrate (1mg/ml pNPP in 1M diethanolamine/1mM $MgCl_2$).

EXAMPLE 5. Production of HC-V biheads by *H. polymorpha*.

5.1 Construction of integration vectors for the expression of anti-hCG/anti-RR6 bispecific biheads.

To allow the expression and secretion of the Llama bihead constructs in *H. polymorpha* strain A16 (Leu-) (Hodgkins et al), the bispecific HC-V gene construct was fused to the alpha-mating factor leader sequence and cloned downstream of the MOX promoter in the *H. polymorpha* transformation and expression vector pHP14.3 (See Figure 17). A culture of *E. coli* cells harbouring plasmid pHP14.3 was deposited under the Budapest treaty at the National collection of Type Cultures (Central Public Health Laboratory) in London (United Kingdom) under deposition number NCTC13048. The

construction of the final expression vectors involved several cloning steps.

Step 1: Construction of the pUC19 based shuttle vector pHP.1 in which the *Hin*dIII/EcoRI polylinker is replaced with a synthetic *Hin*dIII/EcoRI fragment, destroying the original EcoRI site and introducing a BamHI, MunI and two BglII sites:

Synthetic insert of pHP.1:

AAGCTT*AGATCT*GGATCCCGGGCAATTG*AGATCT*AATTC
HindIII BglII BamHI MunI BglII

(see SEQ. ID. NO: 55)

The new insert was constructed by annealing the synthetic oligonucleotides PCR.448 and PCR.449.

PCR.448: 5'-AGCTTAGATCTGGATCCCGGGCAATTGAGATCT-3'

(see SEQ. ID. NO: 56)

PCR.449: 5'-AATTAGATCTCAATTGCCCGGGATCCAGATCTA-3'

(see SEQ. ID. NO: 57)

Step 2: The alpha-mating factor leader-bispecific HC-V gene from the pPIC9-HCV19 and pPIC9-HCV21 vectors were excised as BamHI-EcoRI fragments and inserted into the BamHI/MunI opened shuttle vector pHP.1 giving pHP1.HCV19 and pHP1.HCV21 respectively.

Step 3: In the final cloning step, the NheI/BglII inserts from the intermediate constructs pHP1.HCV19 and pHP1.HCV21 were inserted into the NheI/BglII opened *H. polymorpha*

transformation vector pHP14.3 yielding pHP14.HCV19 and pHP14.HCV21 respectively.

5.2 Production and analysis of the HC-V biheads.

Step 1: Transformation and selection of transformed *H. polymorpha* cells:

H. polymorpha cells (strain A16) were transformed essentially as described under 4.2 except that all culturing was done at 37EC and the pHP constructs were digested with SfiI/NotI before transformation. The plates containing the Leu^+ transformants were used to screen for the Mut^+ and Mut^- phenotype as described under 4.2.

Step 2: Production and evaluation of the bispecific HC-V biheads:

Transformed and selected *H. polymorpha* clones were induced to express bispecific antibody using the same protocol used to express HC-V bihead in *P. pastoris* as described under 4.2. The crude supernatants were tested for the presence of HC-V bihead fragment via analysis on 12% acrylamide gels using the Bio-Rad mini-Protean II system (Figure 18). Bispecific binding activity via shown via ELISA (see 4.2).

EXAMPLE 6. Production of llama HC-V Triple-heads by *S. cerevisiae*.

Upon digesting pUR4603 and pUR4642 with *Bst*EII and *Hin*dIII, the about 6.8kb vector fragment can be isolated and

religated in the presence of the oligonucleotides MPG160WB and MPG161WB (see Example 3.1). From the resulting plasmids, an about 0.4kb *Pst*I fragment can be isolated, encoding the anti-*Streptococcus* HC-V fragments, from which the first five amino acids are lacking at the N-terminus and are fused to the C-terminus. Upon digesting either one of the plasmids pUR4618 - pUR4621 with *Pst*I, and subsequently religating the vectors with the about 0.4 *Pst*I fragments obtained as described above, a new set of yeast expression plasmids can be obtained. The orientation of the about 0.4 *Pst*I fragment in the newly obtained plasmid can be determined via a digestion with *Bst*EII. The proper orientation will result in two fragments of about 0.4kb. The wrong orientation will result in a fragment of about 0.75 and a small fragment of about 0.05kb. Plasmids with the *Pst*I fragment in the proper orientation will encode tripleheads consisting of an HC-V fragment binding to HCG, directly followed by an HC-V fragment binding to *Streptococcus*, directly followed by an HC-V fragment binding to RR6 and finally the myc-tail.

It will be appreciated that it is possible (for those skilled in the art) to design alternative construction routes.

EXAMPLE 7. Reduction of the infectivity of Lactic Acid Bacteria phages by the use of llama HC-V biheads.

7.1 Coupling of RR6-dye molecules to the phage

To coat the phages with RR6 molecules 10 µl P2 phage stock (~10^{12}phages/ml) + 890 µl coupling buffer (0.1M sodium tetraborate, 0.15M NaCl, pH 8.5) + 100 µl RR6 solution in coupling buffer, were mixed and incubated for 1 hour at 37EC (ration of $5*10^4$ RR6 molecules to 1 phage). To inactivate non-reacted chloro-atoms of RR6, 5 µl blocking buffer (1.0M Tris blocking buffer pH 9, with an excess of primary amino groups) was added and incubated 30 minutes at 37EC.

7.2 Effect of biheads on the infectivity of RR6-coated phages.

To test the neutralising effect of the anti-RR6 bihead produced by *S. cerevisiae* containing plasmid pUR4623, this bihead was mixed (in a range of 0-300ng) with $5.5*10^5$ phages in 200 µl total volume and incubated for 0.5 hours at 37EC. From this mixture, 100 µl was added to 100 µl of an overnight culture of *Lactococcus lactis* subsp. *cremoris* LM0230, grown in M17 ($1*10^9$ cfu/ml) and spread on a plate of M17 containing 0.5% glucose and 10mM $CaCl_2$. Plates were incubated overnight at 30EC. In Figure 19 it is shown that 14 ng bivalent antibody fragments give a reduction of infection of over 90%.

EXAMPLE 8. Reduction of the infectivity of Lactic Acid Bacteria (LAB) phages by the use of llama HC-V biheads.

8.1 Raising heavy chain antibodies against the LAB phage P2 and obtaining antigen specific HC-V fragments

An immune response directed against the LAB P2 phages was induced and followed in essentially the same way as described in Example 1. The llama was injected several times with about 0.2 mg phage protein. From the immunised llama an enriched lymphocyte population was obtained and subsequently HC-V gene fragments were obtained as described in Example 2.1. The construction and screening of a yeast HC-V library was performed essentially as described in Examples 2.2 and 2.3.

In this way a number of anti-LAB-phage fragments were obtained. The sequence of three of these are presented below:

pUR3823:
QVQLQESGGG LVQTGGSLRL SCAASGRTSS DYSVGWFRQA PGKEREFLAV MMLSGTGTYY ADSVKGRAAI SRDLAKNTVY LEMNSLKPED TAVYYCALDR AGWLRTEENV YDYWGQGTQV TVSS

(see SEQ. ID. NO: 58)

pUR3824:
QVQLQESGGG LVQPGGSLRL SCAVSGAPFR ESTMAWYRQT PGKERETVAF ITSGGSKTYG VSVQGRFTIS RDSDRRTVLL QMNNLQPEDT AVYYCHRALS NTWGQGIQVT VSS

(see SEQ. ID. NO: 59)

pUR3825:
QVQLQESGGG LVQPGGSLRL SCVVSGEGFS NYPMGWYRQA PGKQRELVAA
MSEGGDRTNY ADAVKGRFTI SRDNAKKTVY LQMSSLKPED TAVYYCNAAR
WDLGPAPFGS WGQGTQVTVS S

(see SEQ. ID. NO: 60)

8.2 Construction of episomal expression plasmids encoding anti-LAB-phage bivalent biheads

Episomal expression plasmids encoding bivalent anti-LAB phage biheads were constructed essentially as described in Examples 3.1 and 3.2, using the above mentioned fragments as starting material. In this way amongst others the plasmids pUR3843 and pUR3850 were constructed encoding the bihead preceded by the SUC2 secretion signal.

pUR3843: SUC2 - 3823 - 3825
pUR3850: SUC2 - 3825 - 3824

The HC-V biheads 3843 and 3850 were produced by yeast transformants obtained essentially as described in Example 3.3.

8.3 Effect of biheads on the infectivity of LAB-phages

To test the neutralising effect of the anti-LAB-phage biheads produced by *S. cerevisiae* containing plasmids pUR3843 or pUR3850, the biheads were mixed (45 µg of 3843 and 24 µg of 3850) with 10^3, 10^6 or 10^8 phages in 200 µl total volume. After an incubation for 0.5 hours at 37EC, 100 µl of an overnight culture of *Lactococcus lactis* subsp. *cremoris* LM0230, grown in M17 ($1*10^6$ cfu/ml) was added to

these mixtures. Subsequently the mixture was spread on a plate of M17 containing 0.5% glucose and 10mM $CaCl_2$. Plates were incubated overnight at 30EC after which the number of pfu's was estimated.

Table 1

Number of plaque forming units

Bihead (µg)	10^3 phages	10^6 phages	10^8 phages
3843 (45)	0	$< 10^3$	Confluent
3850 (24)	0	0	$< 10^3$

8.4 Effect of biheads on the acidification of milk

In a subsequent experiment the acidification of milk upon inoculation with lactic acid bacteria at 30°C was followed by the simultaneous registration of the pH with a HP-3852A Data Acquisition logger. To this end 100 ml XVM-glucose medium (= skim milk containing 0.35% yeast extract, 0.35% peptone and 1% glucose) was inoculated with 100 µl of an overnight culture of *Lactococcus lactis* subsp. *cremoris* LM0230 in XVM-glucose (10^9 cfu/ml) and incubated for 17 h at 30°C after addition of 240 µg bihead 3850. The XVM is acidified by the culture in a period of 4 h (Figure 25, graph 1) and is not influenced by the presence of the bihead. When 10^3 pfu/ml P2 phage was added with the LM0230 culture in a parallel experiment, no acidification occurred during the whole period of 17 h (Figure 25, graph 2). When 10^3, 10^6, or 10^8 pfu/ml P2 phage was added, together with 240 µg bihead 3850, the acidification by the culture can be completely (in the case of 10^3 cfu/ml phage) or partially restored (Figure 25, graphs 3, 4, 5).

EXAMPLE 9. Activating surfaces using HC-V biheads via self assembly, for detection and purification of analytes.

9.1 Use of bispecific HC-V bihead to form an active binding layer on polystyrene wells by self assembling onto pre-adsorbed molecules.

In summary, this example shows how double headed antibody fragments can be used to form an active binding layer on polystyrene wells by self assembling onto pre-adsorbed molecules. Figure 20 shows a diagrammatic representation of this. The adsorbed antibody surface is shown (A) as is a surface sensitised with double headed antibody fragment (B) made by the self assembly of double headed antibody fragment onto a pre-adsorbed RR6-BSA surface (C). These two surfaces are then able to bind hCG (D).

Preparation of a reactive red 6 bovine serum albumin conjugate

A conjugate of reactive red 6 (RR6) and bovine serum albumin (BSA) was made by incubating 200 µl of RR6 (10mg/ml in distilled H_2O) with 1ml of BSA (10mg/ml in phosphate buffered saline) with constant mixing for 3 hours at room temperature. A 200 µl solution of ethanolamine (1M in distilled H_2O) was added and the resulting solution mixed constantly for 15 minutes at room temperature. The BSA-RR6 conjugate was separated from free RR6 by application of 0.75ml to a PD10 column (Pharmacia) and the column eluted with phosphate buffered saline containing 0.1% sodium azide. The eluent was collected as 1ml fractions. Fractions 4 and 5 were red in colour and were pooled (the RR6-BSA conjugate

elutes before unreacted or free RR6). Fractions 10 onwards were also red in colour but these fractions contain the unbound RR6 and so were discarded.

Preparation of polystyrene wells adsorbed with RR6-BSA

Individual wells from Greiner high binding plates were isolated from a 96 well plate by sawing. Into these wells, 100 µl aliquots of 10 µg/ml of RR6-BSA or monoclonal antibody recognising hCG was added and incubated for 3 hours at room temperature. Both the antibody adsorbed and RR6-BSA adsorbed wells were then washed 3 times with PBSTA.

Self assembly of a double headed antibody fragment onto RR6-BSA wells

Following the PBSTA wash of RR6-BSA wells, 100 µl of affinity purified double headed antibody fragment (HI15-R9: pPIC.HCV21) at 20 µg/ml in PBSTA was added per well. After 1 hour the RR6-BSA wells were washed 3 times with PBSTA.

Capture of I^{125} labelled hCG to antibody adsorbed and double headed antibody fragment sensitised RR6-BSA wells

hCG (2500 IU/ml in PBSTA) was spiked with 10 µCi I^{125} hCG (Amersham) and then diluted to 500, 100, 20 and 4 IU/ml of hCG with PBSTA. Aliquots (100 µl) of these dilutions were incubated in the wells for 1 hour at room temperature after which time the wells were extensively washed with PBSTA. Wells were then counted on a gamma counter. Figure 21 shows the amount of dpm captured by the monoclonal antibody adsorbed wells (A) and by the double headed antibody fragment sensitised wells (B) over a range of hCG concentrations.

The double headed antibody fragment sensitised wells (HI15-R9: pPIC.HCV21) bound approximately twice as much hCG than the adsorbed antibody wells at saturating hCG concentrations. This would indicate that the double headed antibody fragment sensitised wells possessed a higher density of active hCG binding sites than the adsorbed antibody wells.

9.2 Use of bispecific HC-V bihead to self assemble on latex particles for the detection of human chorionic gonadotrophin (hCG)

Preparation of a RR6-BSA latex

To 950 µl of 10mM borate buffer, 0.01% merthiolate, pH 8.5 a 50 µl aliquot of Duke blue latex (10% solids) was added and mixed by inverting. The diluted latex was then centrifuged at 8,000 g for 10 minutes at room temperature, the supernatant removed and the pellet vortexed briefly. The pellet was re-suspended in a solution made up of 900:1 of borate buffer (as above) and 100 µl of the previously prepared RR6-BSA conjugate. Latex particles were sonicated for 10 s using a sonic probe. The solution containing the latex was mixed for 30 minutes at room temperature. Following this the latex was pelleted as before and re-suspended in 1 ml of the borate buffer.

Self assembly of a double headed antibody fragment onto RR6-BSA latex

By virtue of its specificity the double headed antibody fragment (HI15-R9: pPIC.HCV21) self assembles on the surface of a RR6-BSA latex. This was achieved by incubating 5:1 of the RR6-BSA latex with 3:1 of supernatant from *Pichia*

pastoris expressing the bihead HI15-R9 (pPIC.HCV21) made up to 40 µl with phosphate buffered saline containing 0.1% sodium azide and 0.1% Tween 20 (PBSTA) for 15 minutes at room temperature.

Assessing the self assembled double head for use in hCG assay

hCG (10 µl at various concentrations) was added to the self assembled double head RR6-BSA latex and incubated for 15 minutes at room temperature. The mixture was then allowed to flow up a nitrocellulose strip. The nitrocellulose strip had a line of antibody recognising a different epitope to hCG than the double headed antibody fragment (Figure 22 shows a schematic representation of the principle). The amount of latex binding at the antibody line was determined by scanning the intensity of the line using an autoreader. The results are shown in Figure 23. By way of comparison a latex was made by adsorption of a monoclonal antibody, specific for hCG, using a similar methodology as that for the RR6-BSA latex. This latex was incubated with hCG (10 µl at the various concentrations) and also subjected to the same evaluation on a nitrocellulose strip (results shown in Figure 23).

Figure 23 shows that the self assembling latex compares well with the adsorbed antibody latex. In fact, the hook effect seen with the adsorbed latex (labelled A in Figure 23) at the higher hCG concentrations is less pronounced with the self assembling latex (labelled B in Figure 23) giving the assay a higher range of hCG detection. The most likely explanation for this is an increased number of hCG binding

sites on the self assembling latex compared with the adsorbed latex.

9.3 Assembly of bispecific HC-V antibody fragments to a dextran surface coupled with RR6-BSA conjugate

Preparation of a Bialite chip coupled with RR6-BSA

A new CM5 biosensor chip (Biacore AB) was docked into a Bialite biosensor (Biacore AB). The flow rate, of HEPES buffered saline (HBS) was set to 10 µl/min. The RR6-BSA conjugate was amine coupled to the CM5 chip using an amine coupling kit (Biacore AB) according to the manufacturer's instructions. Briefly, two 40 µl injections of NHS/EDC were performed to activate the biosensor chip surface. Following activation, two 40 µl injections of RR6-BSA (diluted 1:10 in 10mM sodium acetate, pH 4.0) were performed. The biosensor chip surface was then blocked by two injections of 40:1 ethanolamine (1M).

Preparation of a binding surface by self assembly of double headed antibody fragment and subsequent detection of hCG

Double headed antibody fragment (HI15-R9: pPIC.HCV21) was assembled onto the RR6-BSA coupled dextran surface by injection of 20 µl purified double headed antibody fragment (HI15-R9: pPIC.HCV21, 100 µg/ml in HBS). This can be seen as an increase in response units (RU) on the Bialite sensorgram shown in Figure 24 labelled A. hCG was then injected (20 µl of a 10 IU/ml solution made up in HBS). Detection of hCG can be seen by an increase in RU (Figure 24, labelled B).

EXAMPLE 10. Anti-hCG/anti-RR6 bispecific biheads containing a linker peptide.

10.1 Construction of *S. cerevisiae* episomal expression plasmids encoding anti-hCG/anti-RR6 bispecific biheads containing a linker peptide.

Between the H14 and the R9 encoding DNA fragments synthetic linkers were introduced encoding different linker peptides. To this end the about 50 bp long *Bst*EII-*Hin*dIII fragment of pJS7 (see Example 3.1) was replaced by an about 50 bp long *Bst*EII-*Hin*dIII fragment having the following sequence:

MVaJA

*Bst*EII *Xba*I *Dra*III *Pst*I *Hin*dIII

5' **GTCACC**GTC**TCTAGA**TGGC**CACCAGGTG**CAG**CTGCAG**GAGTCAACTT**A** 3'

(see SEQ. ID. NO: 61)

MVbJA

3' G**CAGAGATCT**ACCG**GTGGTCCAC**GTC**GAGCTC**CTCAGTTGAA**TTCGA** 5'

(see SEQ. ID. NO: 62)

This resulted in pSJ7a. In this plasmid the about 20 bp *Pst*I-*Hin*dIII fragment was replaced with the about 370 bp *Pst*I-*Hin*dIII fragment encoding the anti-RR6 HC-V fragment R9 and/with the myc-tail of pUR4640 (see Example 3.1) and resulting in pSJ7b.

Upon digesting plasmid pSJ7b with *Xba*I and *Dra*III the about 7 kb vector fragment was ligated with five synthetic oligo nucleotide linker fragments presented below:

MV01JA 5′ CTAGTGGTACTTCCGGTTCCCAG 3′
(see SEQ. ID. NO: 63)

MV02JA 3′ ACCATGAAGGCCAAGG 5′
(see SEQ. ID. NO: 64)

S **G T S G S** Q
(see SEQ. ID. NO: 65)

MV03JA 5′ CTAGTTCTTCATCTGCTTCTGCCTCTTCAGCCCAG 3′
(see SEQ. ID. NO: 66)

MV04JA 3′ AAGAAGTAGACGAAGACGGAGAAGTCGG 5′
(see SEQ. ID. NO: 67)

S **S S S A S A S S A** Q
(see SEQ. ID. NO: 68)

MV05JA 5′CTAGTGGTTCTCCAGGTTCACCAGGTCAG 3′
(see SEQ. ID. NO: 69)

MV06JA 3′ ACCAAGAGGTCCAAGTGGTCCA 5′
(see SEQ. ID. NO: 70)

S **G S P G S P G** Q
(see SEQ. ID. NO: 71)

MV07JA 5′ CTAGTGCTACTACAACTGGTTCTTCACCAGGTCCAACTCAG 3′
(see SEQ. ID. NO: 72)

MV08JA 3′ ACGATGATGTTGACCAAGAAGTGGTCCAGGTTGA 5′
(see SEQ. ID. NO: 73)

S **A T T T G S S P G P T** Q
(see SEQ. ID. NO: 74)

MV09JA 5′ CTAGTGCTAATCATTCTGGTAATGCTTCTCAG 3′
(see SEQ. ID. NO: 75)

MV10JA 3′ ACGATTAGTAAGACCATTACGAAGA 5′
(see SEQ. ID. NO: 76)

S **A N H S G N A S** Q
(see SEQ. ID. NO: 77)

The oligonucleotide linker fragments encode the last amino acid of the N-terminal HC-V fragment (S) and the first amino acid of the C-terminal HC-V fragment, intersected by the connecting linker peptide. This resulted in plasmids pUR5330 to 5334, respectively.

After transformation of *S. cerevisiae* with these plasmids, the production levels of the biheads were determined via Western blot analysis and a anti-hCG ELISA using anti-myc mAb for detection of the bound bihead (see Example 2.3). Production levels are presented in Table 2 below:

Table 2

Plasmid	Linker	Production level (mg/l)
pUR4619	None	11
pUR5330	S-**G-T-S-G-S**-Q	36
pUR5331	S-**S-S-S-A-S-A-S-S-A**-Q	49
pUR5332	S-**G-S-P-G-S-P-G**-Q	33
pUR5333	S-**A-T-T-T-G-S-S-P-G-P-T**-Q	56
pUR5334	S-**A-N-H-S-G-N-A-S**-Q	51

The production levels of the biheads in which the two HC-V domains are separated by a linker peptide (consisting of between 5 and 11 amino acids) were found to be 3 to 5 times higher as found for the bihead in which the two HC-V fragments are connected without a peptide linker.

It is therefore expected that other linker peptides, e.g. the short hinge regions found in the heavy chain antibodies

are equally suitable and give even better production yields.

Finally, the bispecificity of the biheads was demonstrated using the ELISA as described in Example 3.3, the results of which are presented in Figure 26.

Literature referred to in the Examples

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Harmsen, M.M., Langedijk, A.C., van Tuinen, E., Geerse, R.H., RauP, H.A., Maat, J., (1993) Effect of pmr1 disruption and different signal sequences on the intracellular

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO Dr Paul van der Logt
Head of BioRecognition Unit
Unilever Research
Colworth House
Sharnbrook
BEDFORD
MK44 1LQ
NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: p HP 14.3a in E. coli XL-1 Blue	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 13048

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 26th August 1997 (date of original deposit) [1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: National Collection of Type Cultures Address: Central Public Health Laboratory 61 Colindale Avenue LONDON, NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 18/11/97 Barry Holmes B Holmes Clinical Scientist

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

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FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Unilever Research Laboratorium Vlaardingen
Postbus 114
3130 AC VLAARDINGEN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
E. coli JM109-pUR4547	CBS 100012

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary accepts the microorganism identified under I above, which was received by it on Monday, 18 August 1997 *(date of the original deposit)*[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on not applicable *(date of the original deposit)* and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable *(date of receipt of request for conversion)*

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
P.O. Box 273
3740 AG BAARN
The Netherlands

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Mrs F.B. Snippe-Claus dr R.A. Samson

Date: Friday, 29 August 1997

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Unilever Research Laboratorium Vlaardingen
Postbus 114
3130 AC VLAARDINGEN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
E. coli JM109-pUR4548	CBS 100013

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(mark with a cross where applicable)

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Date: Friday, 29 August 1997

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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PBS 9147

Claims

1. A multivalent antigen binding protein comprising a single polypeptide chain comprising, in series, two or more single domain binding units.

2. A protein according to claim 1, wherein the single domain binding units are heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains.

3. A protein according to claim 1 or claim 2, wherein the single domain binding units are heavy chain variable domains derived from a Camelid immunoglobulin.

4. A protein according to any ones of claims 1 to 3, comprising a bivalent antigen binding protein.

5. A protein according to any one of claims 1 to 4, wherein the single domain binding units are connected directly in series.

6. A protein according to any one of claims 1 to 4, wherein the single domain binding units are connected in series by a peptide linker.

7. A protein according to any one of claims 1 to 6, wherein the protein has an additional peptide appended to it.

8. A protein according to any one of claims 1 to 7, wherein the protein has an enzyme fused to it.

9. A protein according to any one of claims 1 to 8, wherein the single domain binding units have different antigen specificity from each other.

10. A protein according to any one of claims 1 to 8, wherein the single domain binding units have the same antigen specificity as each other.

11. Nucleotide sequences coding for the polypeptide of the multivalent antigen binding protein of any one of the preceding claims.

12. An expression vector comprising a nucleotide sequence according to claim 11.

13. A host cell transformed with a vector according to claim 12 and capable of expression of the nucleotide sequences to produce the polypeptide of the multivalent antigen binding protein.

14. A process for preparing a multivalent antigen binding protein according to any one of claims 1 to 10 comprising transforming a host by incorporating a gene encoding said polypeptide and expressing said gene in a host.

15. Use of a multivalent antigen protein according to any of claims 1 to 10 in diagnosis, therapy, targeting, immunoassays, cross-linking methods, including agglutination, or for purification processes.

16. Use of a multivalent antigen protein according to any one of claims 1 to 10, for inactivation of (bacterio)phages or viruses.

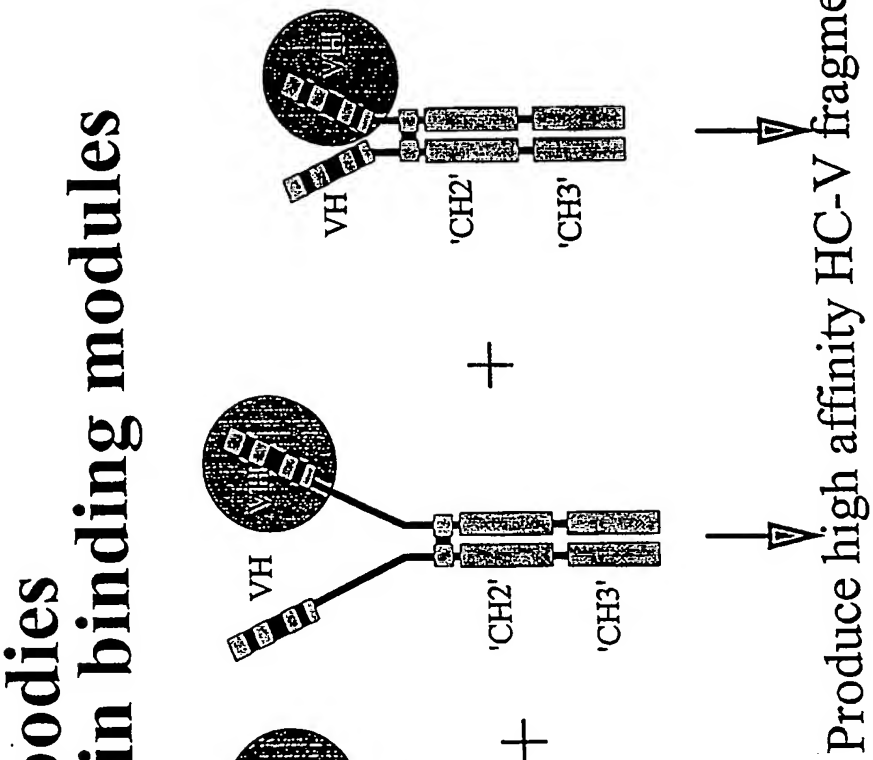


Figure 1. Schematic representation of Camelidae IgG types

Figure 2.

pUR 4638 Anti-RR6 R 7

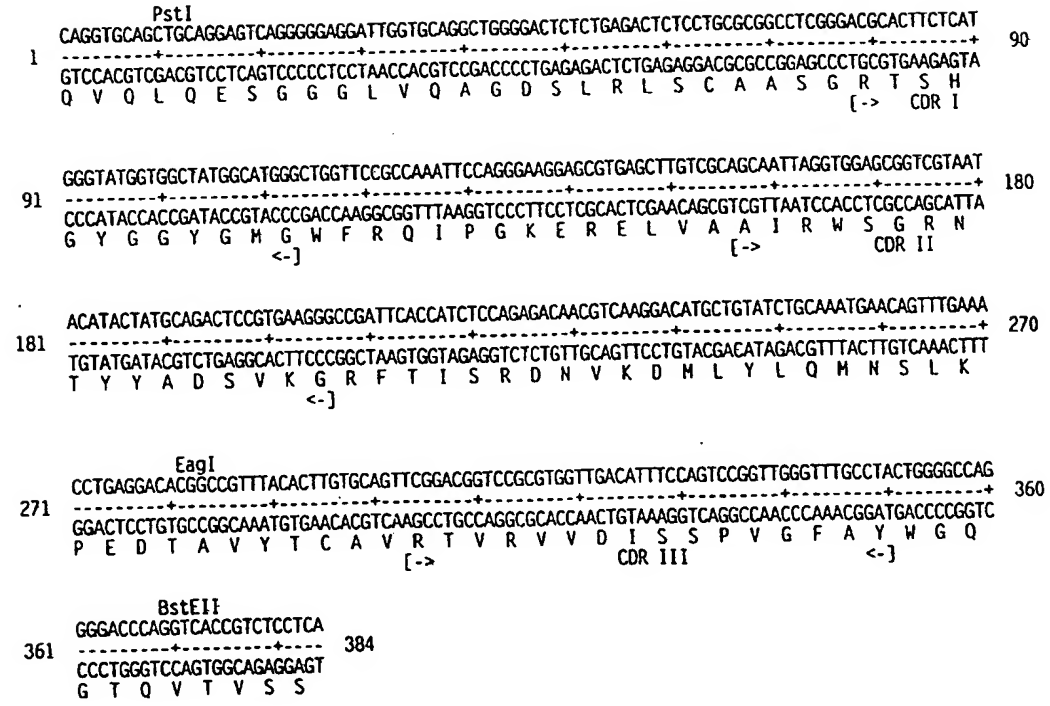


Figure 3.

pUR 4640 Anti-RR6 R9

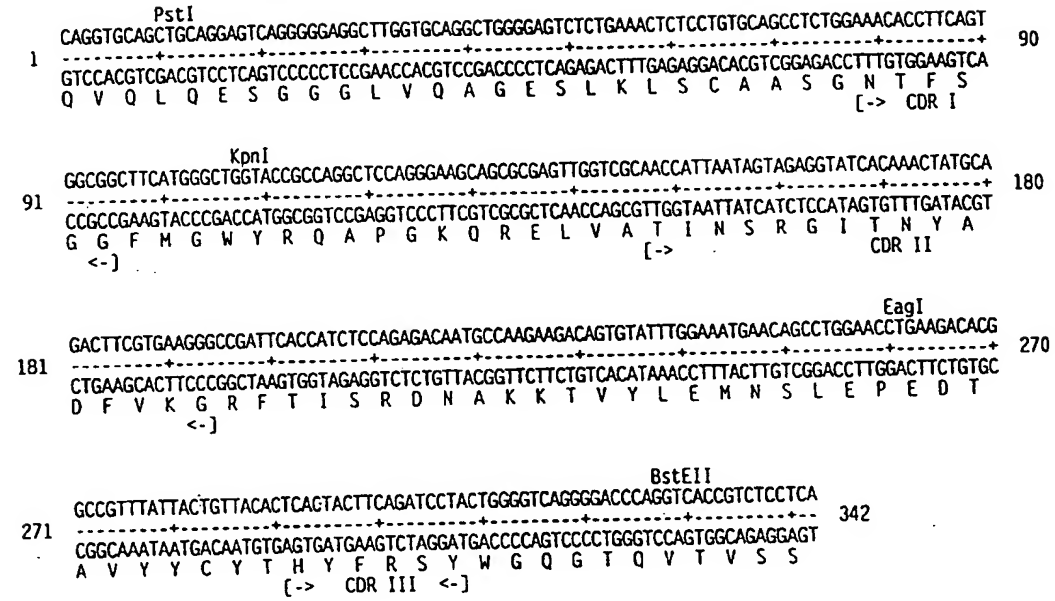
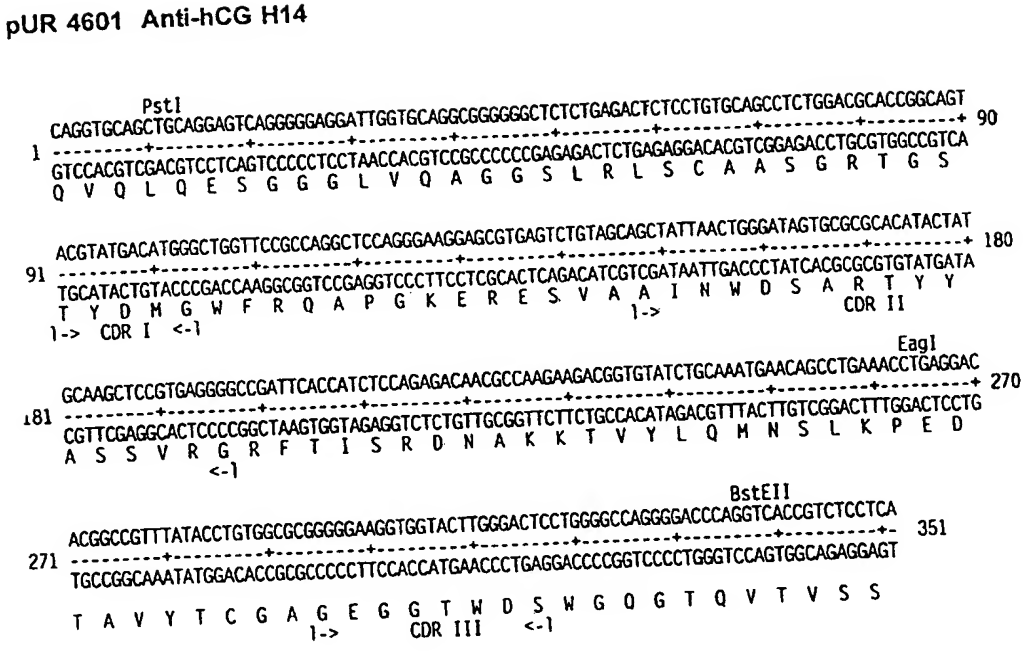


Figure 4.


pUR 4601 Anti-hCG H14
PstI
CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGGCGGGGGGCTCTCTGAGACTCTCCTGTGCAGCCTCTGGACGCACCGGCAGT
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Q V Q L Q E S G G G L V Q A G G S L R L S C A A S G R T G S
ACGTATGACATGGGCTGGTTCCGCCAGGCTCCAGGGAAGGAGCGTGAGTCTGTAGCAGCTATTAACTGGGATAGTGCGCGCACATACTAT
TGCATACTGTACCCGACCAAGGCGGTCCGAGGTCCCTTCCTCGCACTCAGACATCGTCGATAATTGACCCTATCACGCGCGTGTATGATA
T Y D M G W F R Q A P G K E R E S V A A I N W D S A R T Y Y
1-> CDR II
1-> CDR I <-1
EagI
GCAAGCTCCGTGAGGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAAGACGGTGTATCTGCAAATGAACAGCCTGAAACCTGAGGAC
CGTTCGAGGCACTCCCCGGCTAAGTGGTAGAGGTCTCTGTTGCGGTTCTTCTGCCACATAGACGTTTACTTGTCGGACTTTGGACTCCTG
A S S V R G R F T I S R D N A K K T V Y L Q M N S L K P E D
<-1
BstEII
ACGGCCGTTTATACCTGTGGCGCGGGGGAAGGTGGTACTTGGGACTCCTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
TGCCGGCAAATATGGACACCGCGCCCCCTTCCACCATGAACCCTGAGGACCCCGGTCCCCTGGGTCCAGTGGCAGAGGAGT
T A V Y T C G A G E G G T W D S W G Q G T Q V T V S S
1-> CDR III <-1

Figure 5.

pUR 4602 Anti-hCG HI - 15

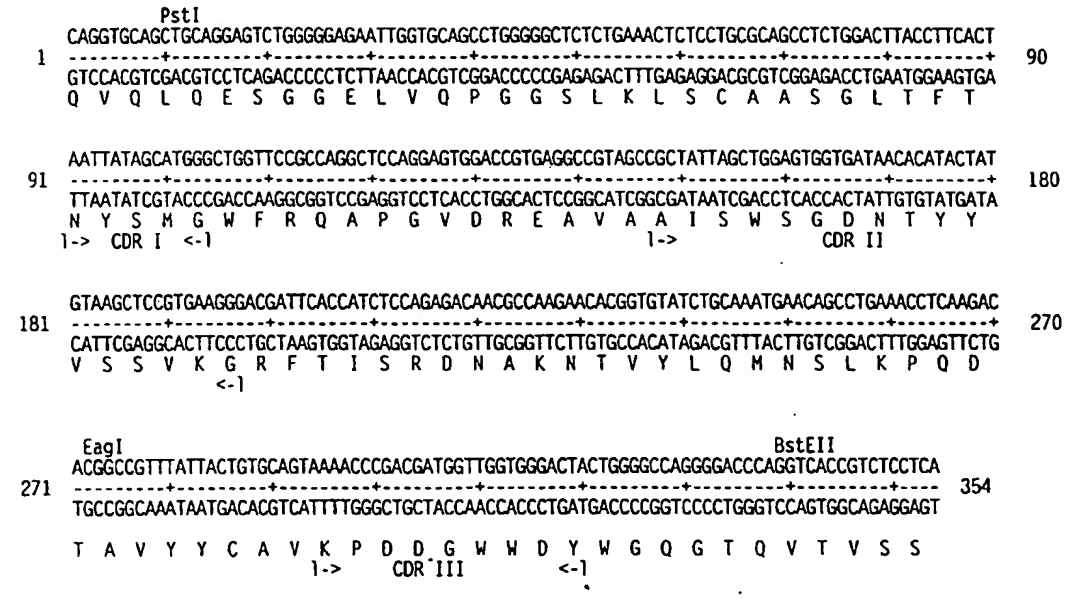


Figure 6.

pUR 4603 Anti-*Streptococcus* S36

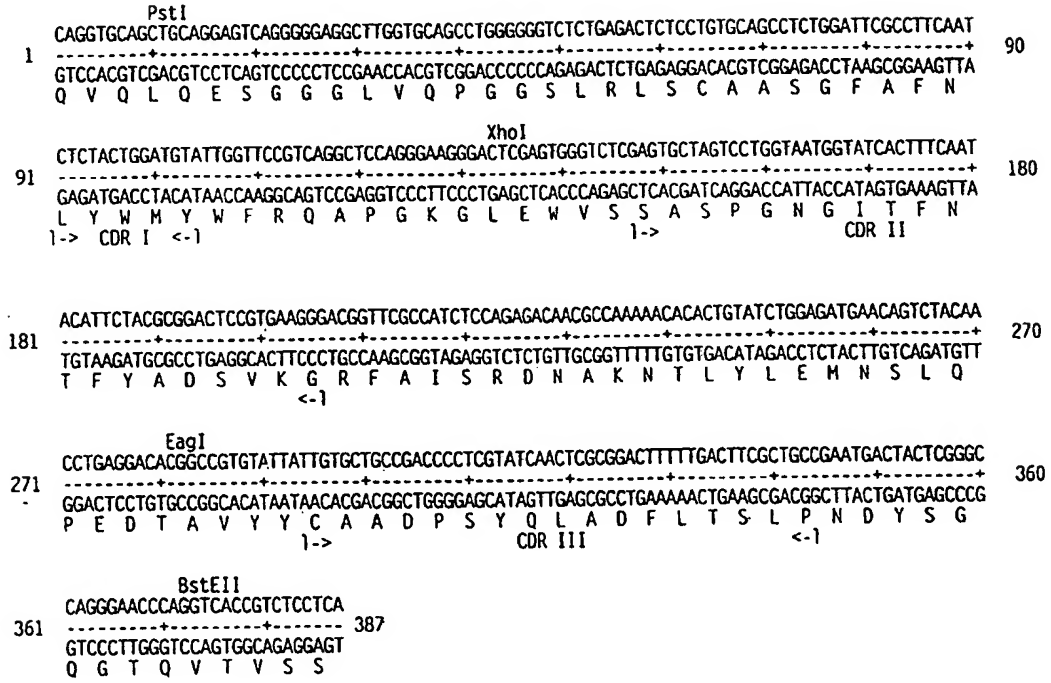
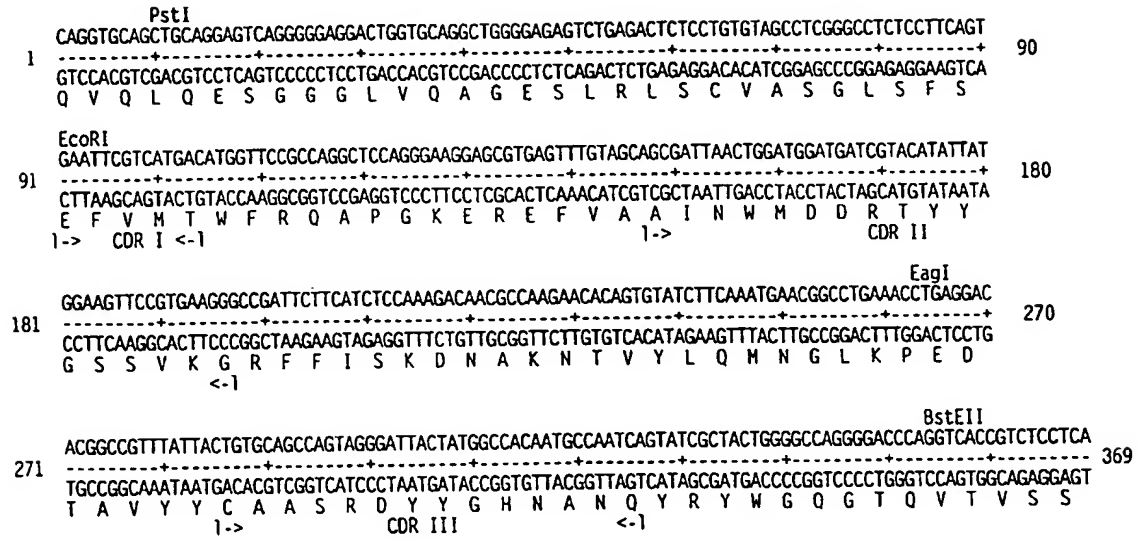


Figure 7.

pUR 4642 Anti-*Streptococcus* S120



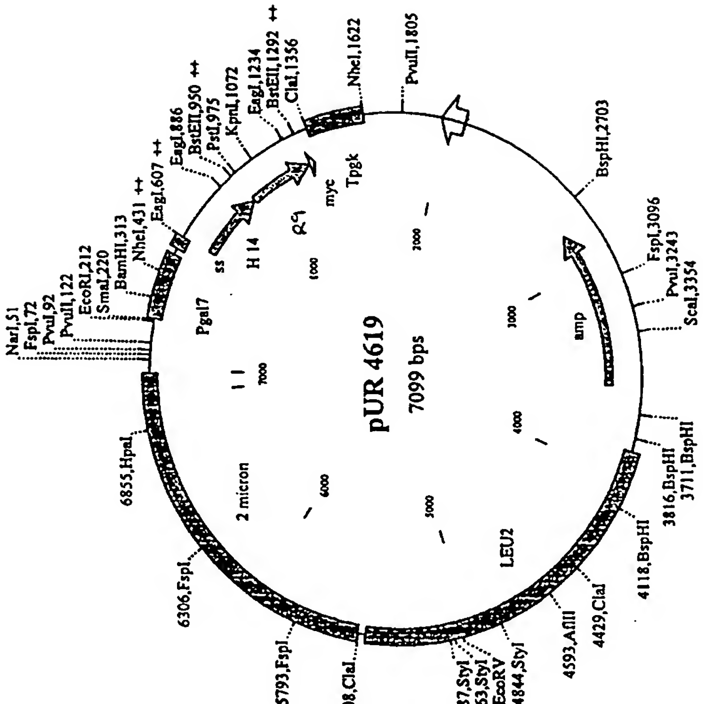

pUR 4619
7099 bps
NarI,51
FspI,72
PvuI,92
PvuII,122
EcoRI,212
SmaI,220
BamHI,313
NheI,431 ++
EagI,607 ++
EagI,886
BstEII,950 ++
PstI,975
KpnI,1072
EagI,1234
BstEII,1292 ++
ClaI,1356
NheI,1622
PvuII,1805
BspHI,2703
FspI,3096
PvuI,3243
ScaI,3354
3711,BspHI
3816,BspHI
4118,BspHI
4429,ClaI
4593,AflII
4844,StyI
5026,EcoRV
5063,StyI
5087,StyI
5508,ClaI
5793,FspI
6306,FspI
6855,HpaI
Pgal7
ss
H 14
myc
Tpgk
amp
LEU2
2 micron
1000
2000
3000
4000
5000
6000
7000

Figure 8.

Figure 9.

pUR 4619 (Bihead H14 - R9)

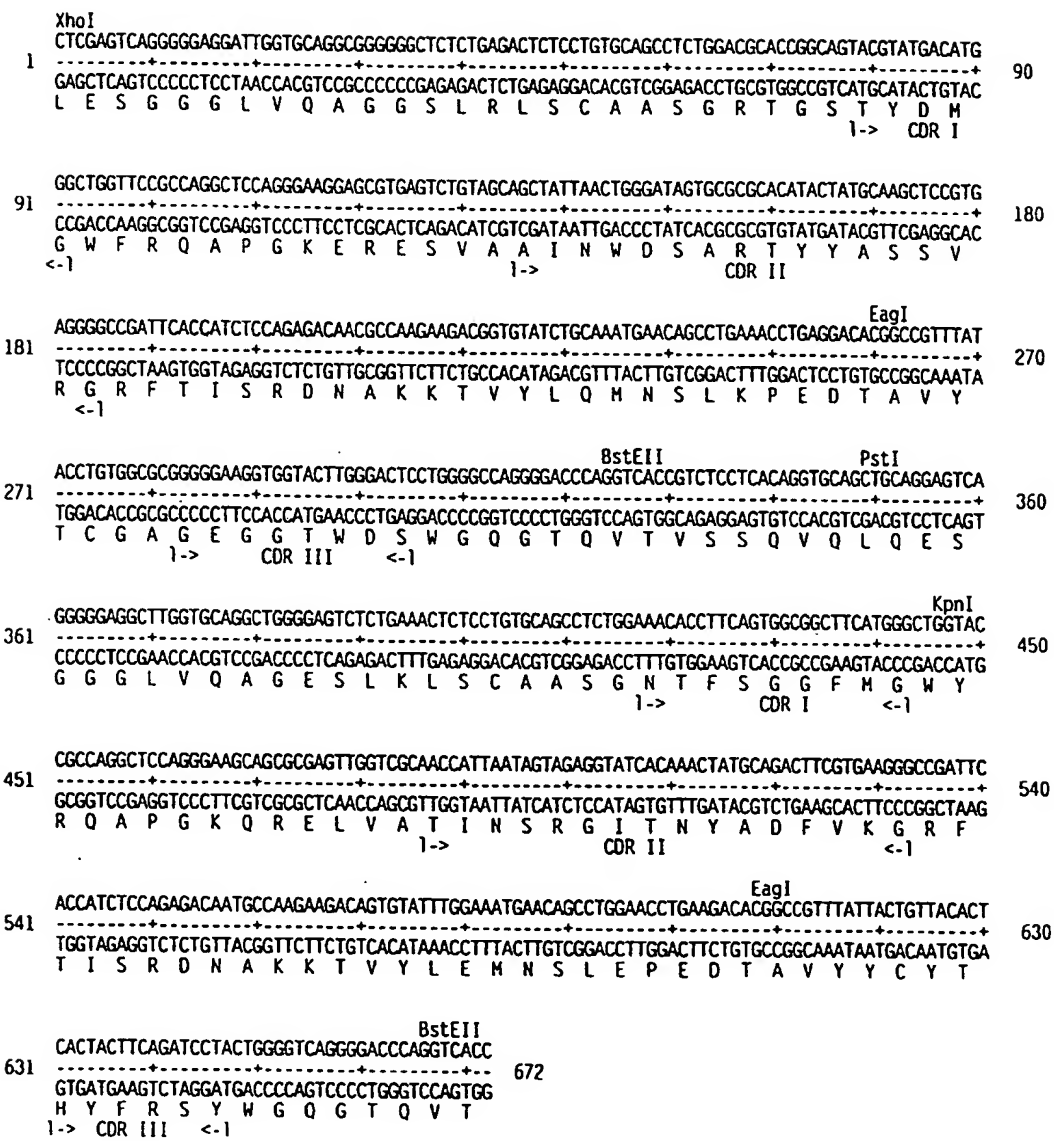


Figure 10.

pUR 4620 (Bihead HI 15 - R7)

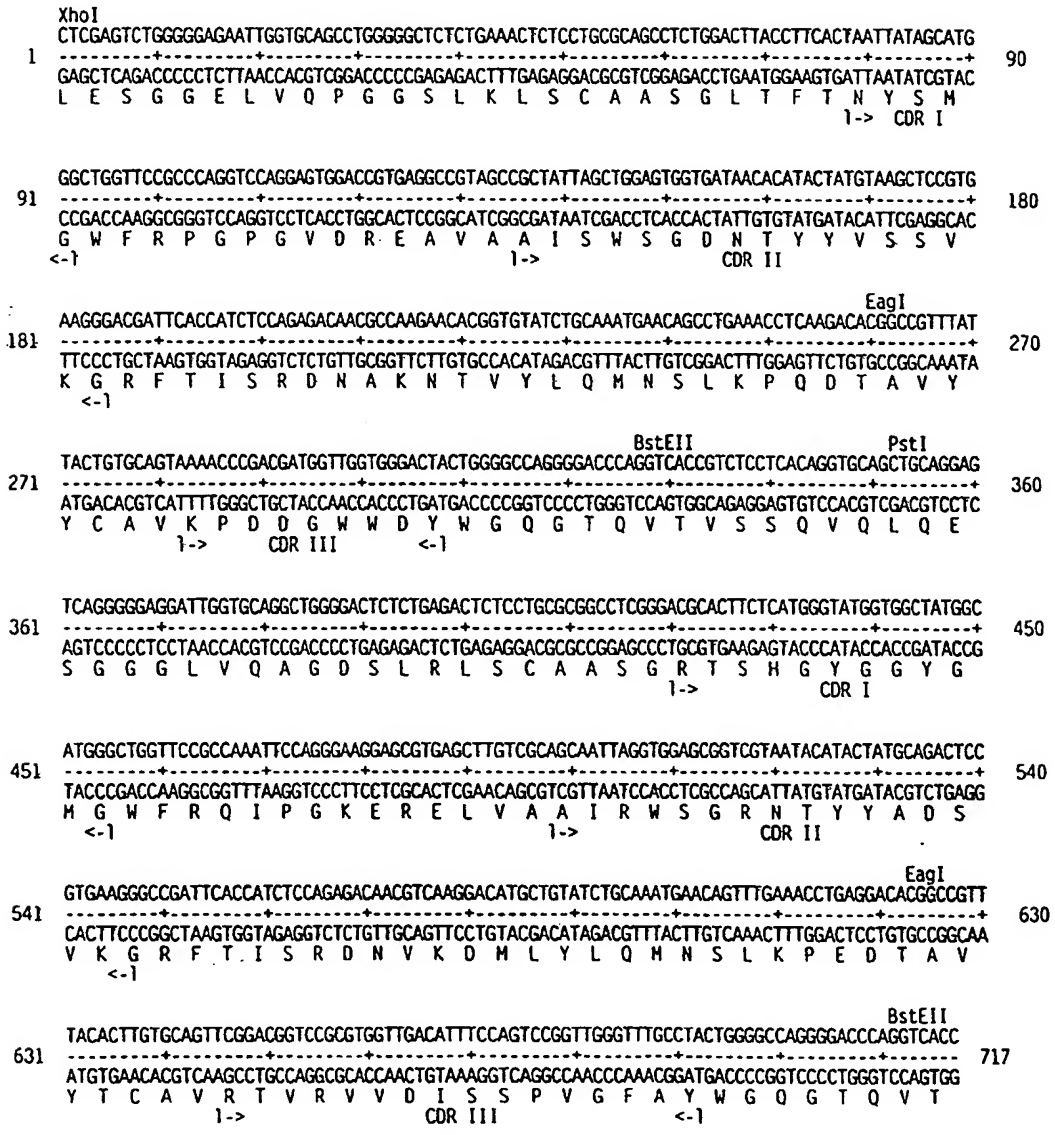


Figure 11.

pUR 4621 (Bihead HI 15 - R9)

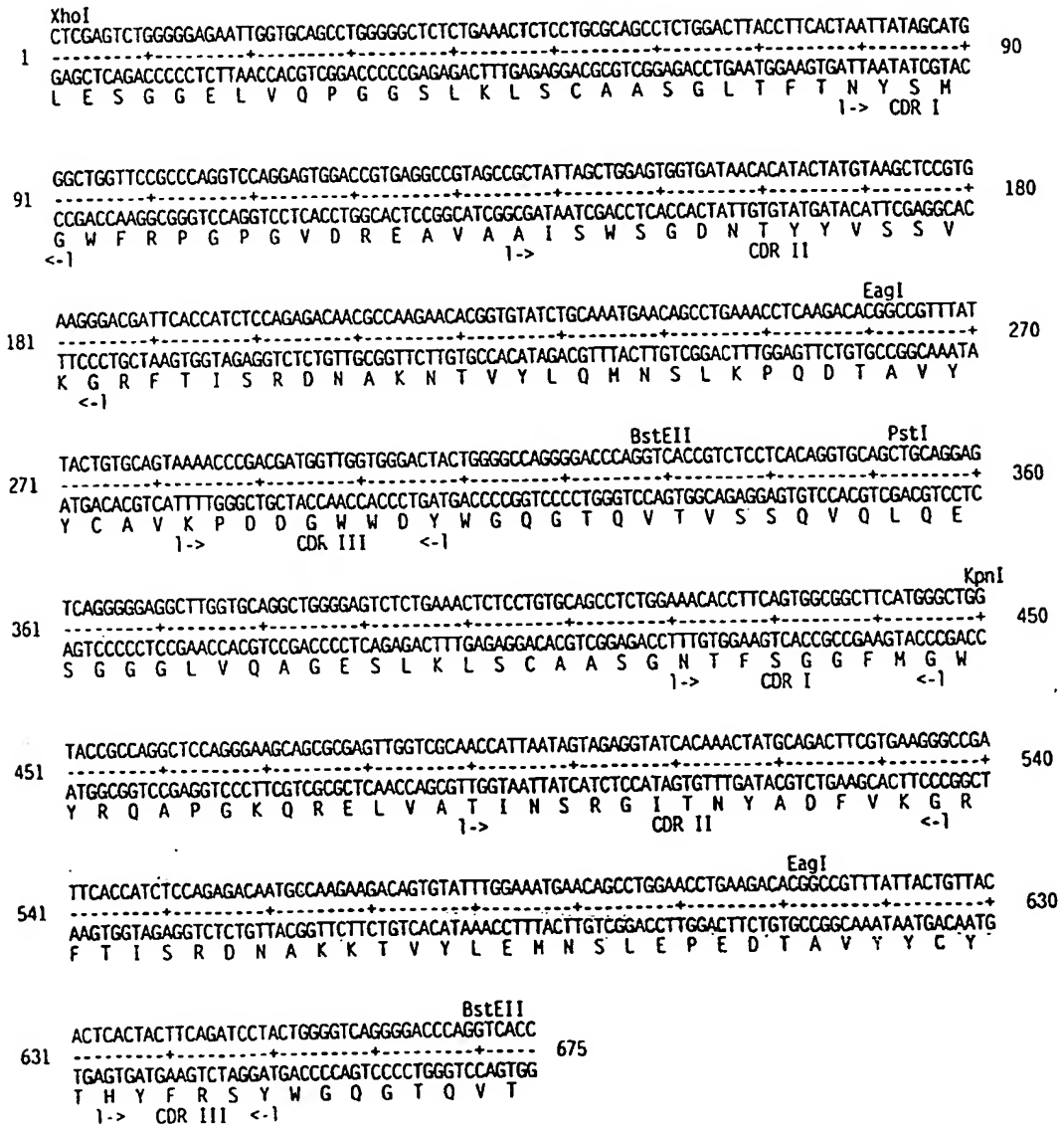


Figure 12.

pUR 4622 (Bihead R7 - R7)

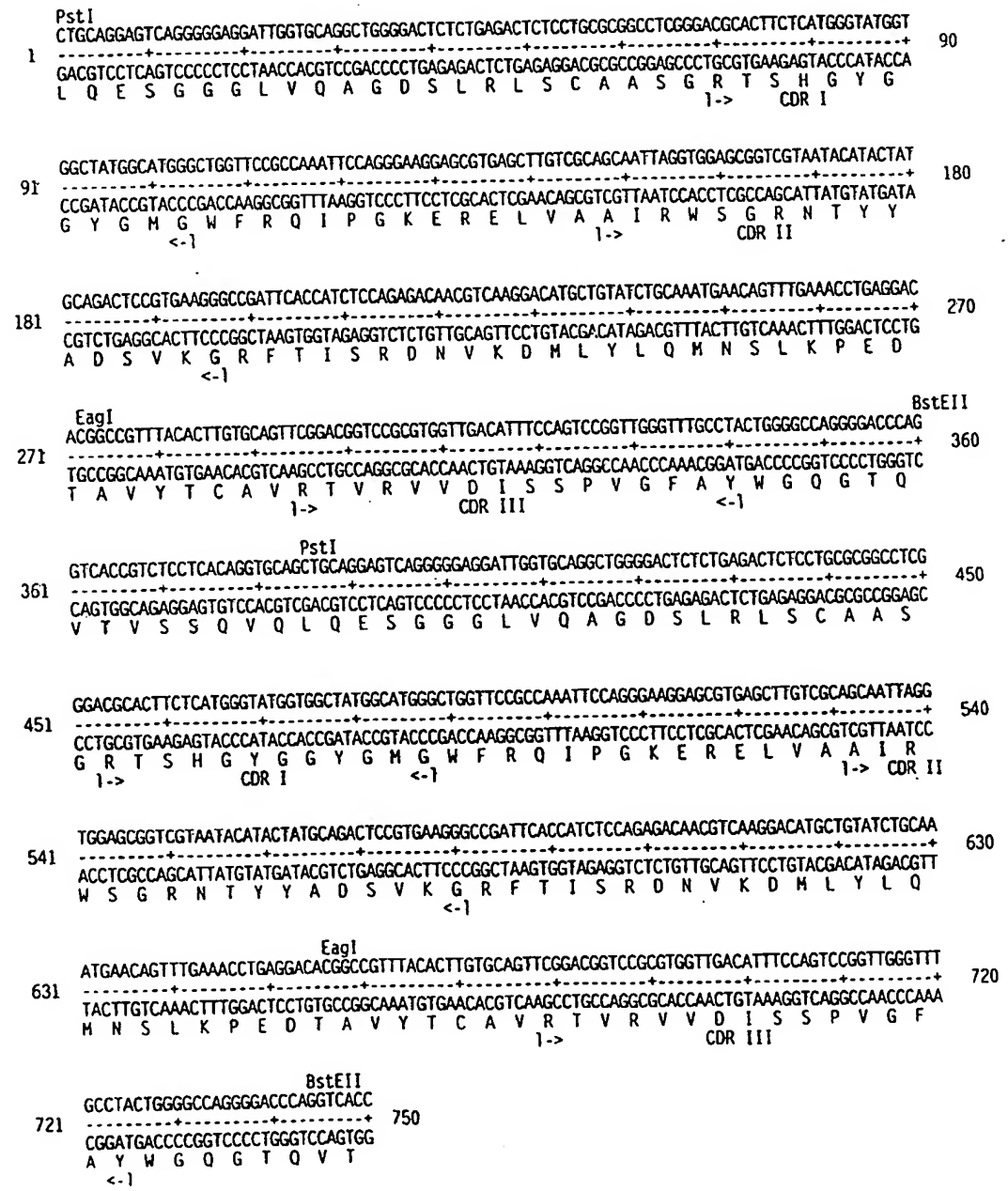
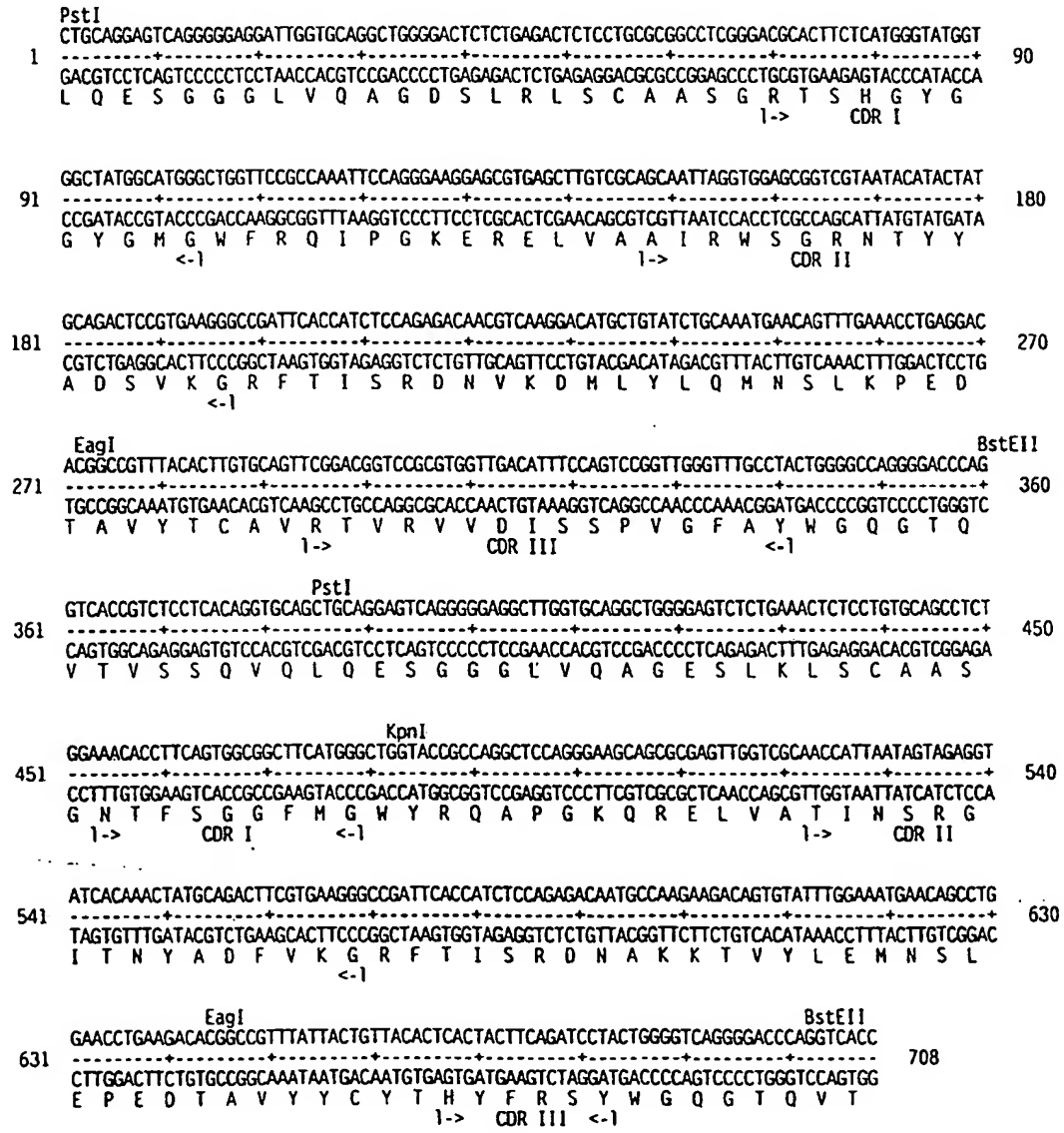


Figure 13.

pUR 4623 (Bihead R7 - R9)



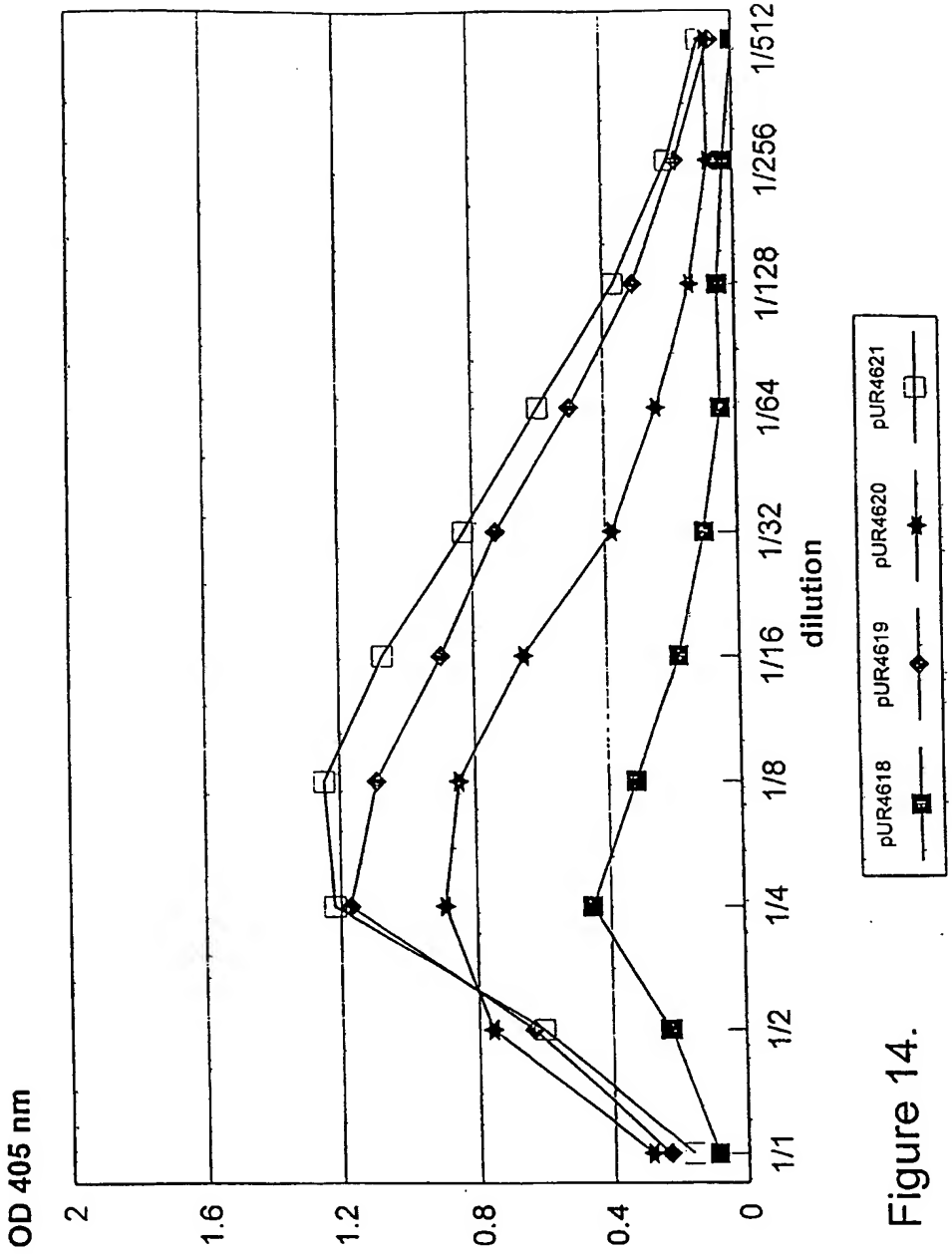

HCG - RR6 ELISA
OD 405 nm
2
1.6
1.2
0.8
0.4
0
1/1
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
dilution
pUR4618
pUR4619
pUR4620
pUR4621

Figure 14.

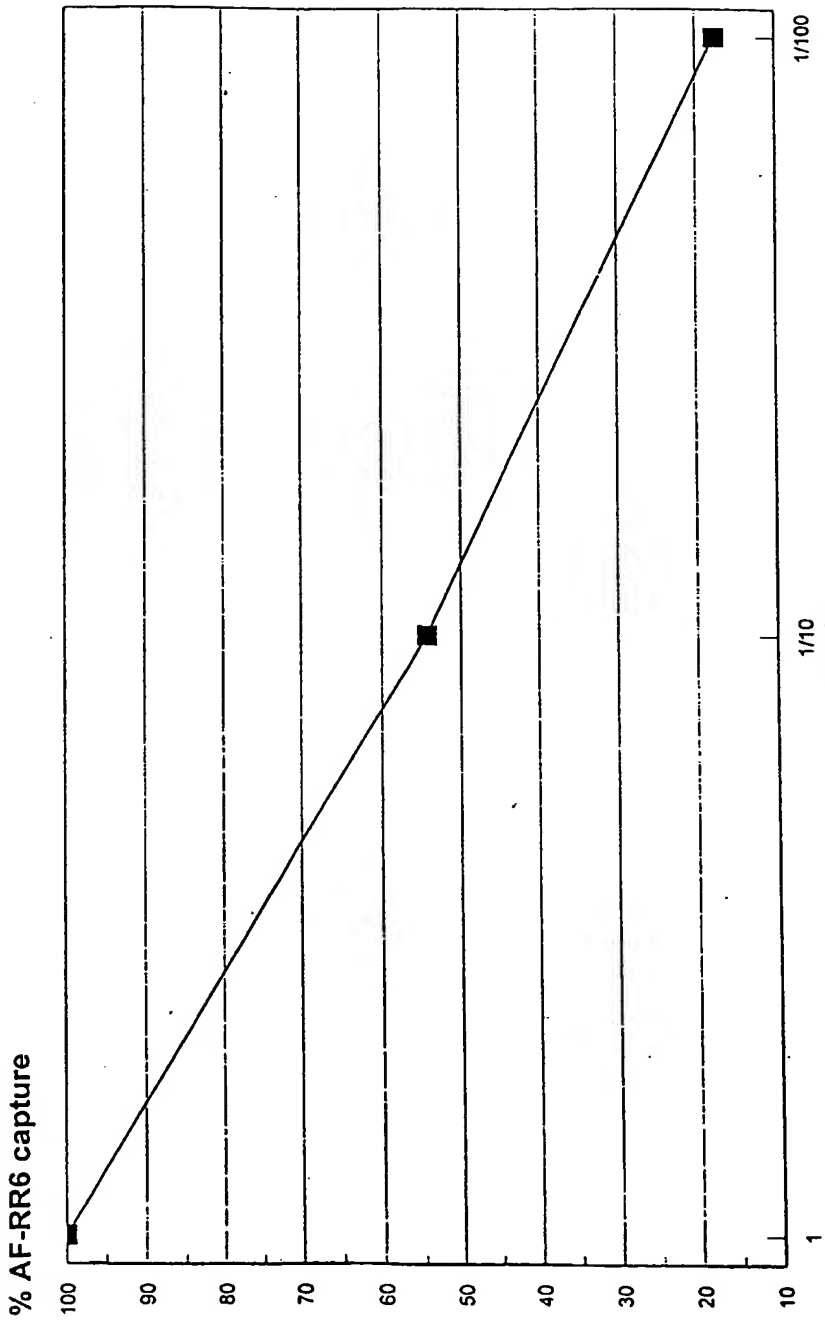


Figure 15. Results of a RR6/RR6 bifunctional binding assay

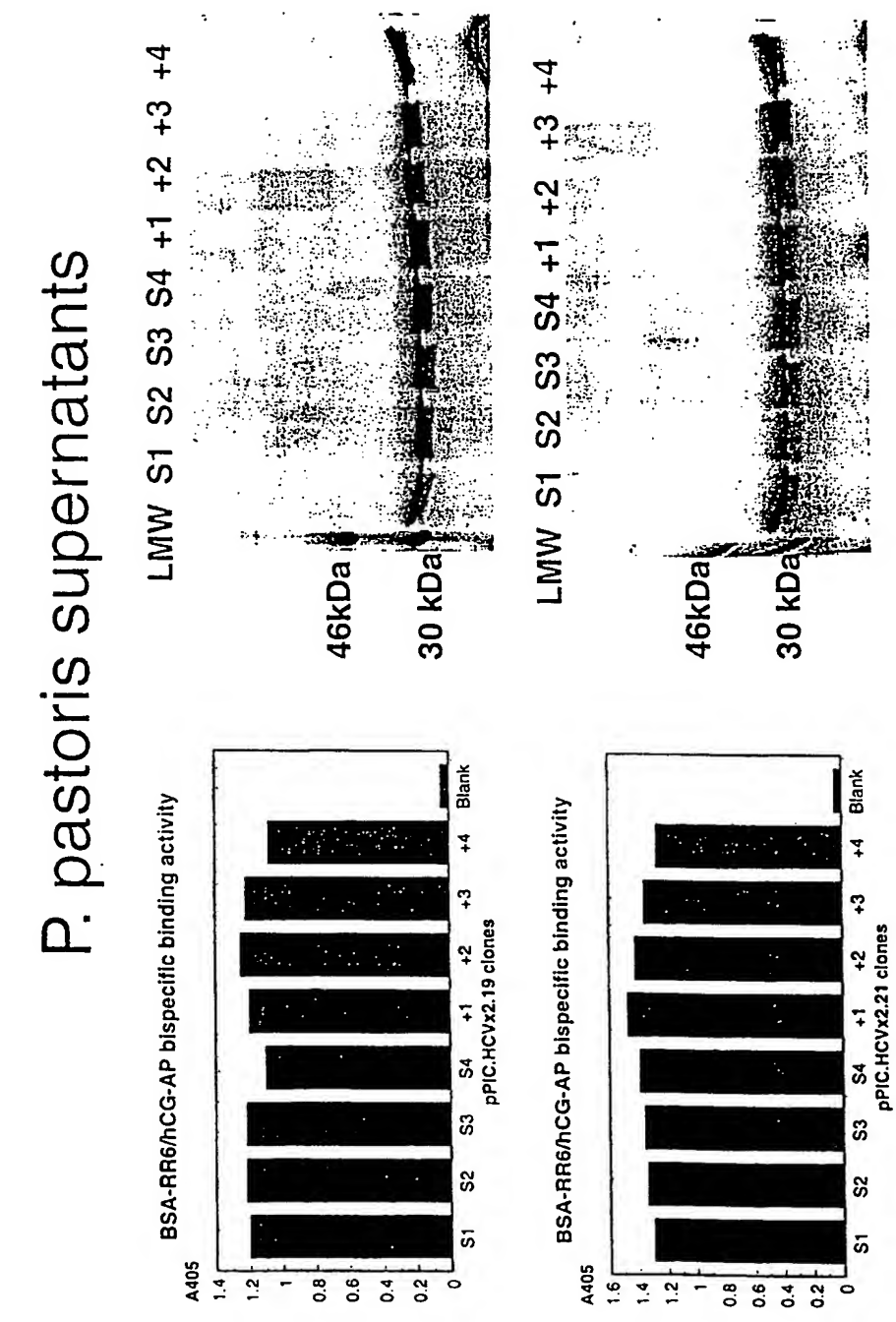


Figure 16 ELISA and SDS-Page analysis of Crude *P. pastoris* supernatants expressing HC-V bihead

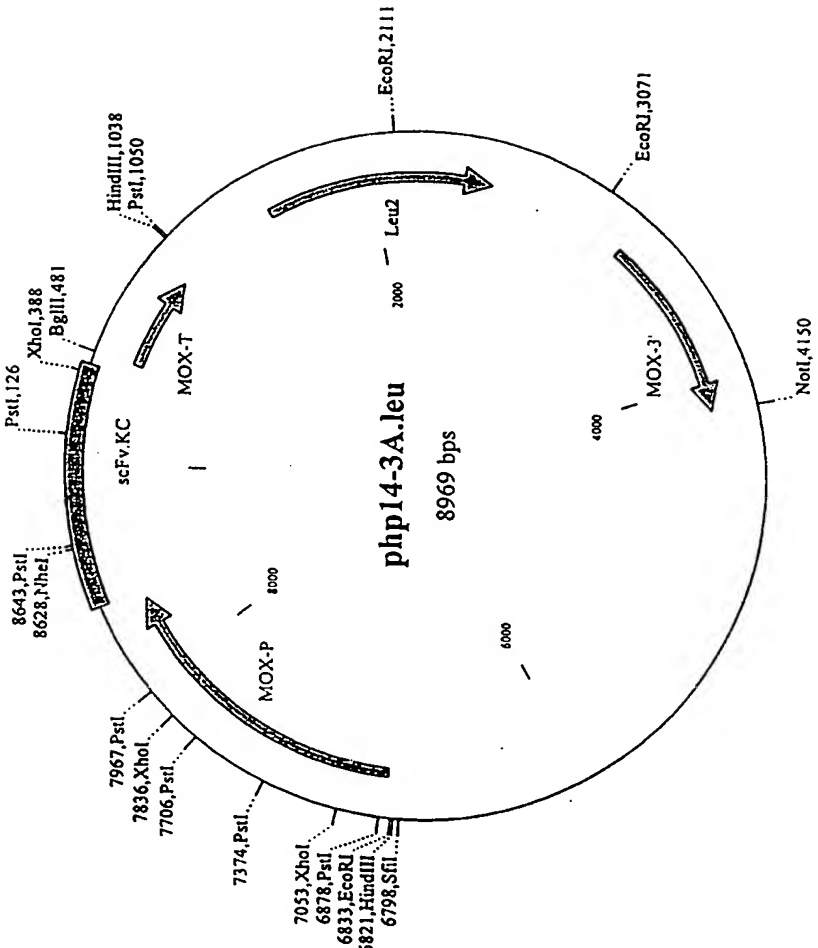


Figure 17. Plasmid map of pHP14.3A

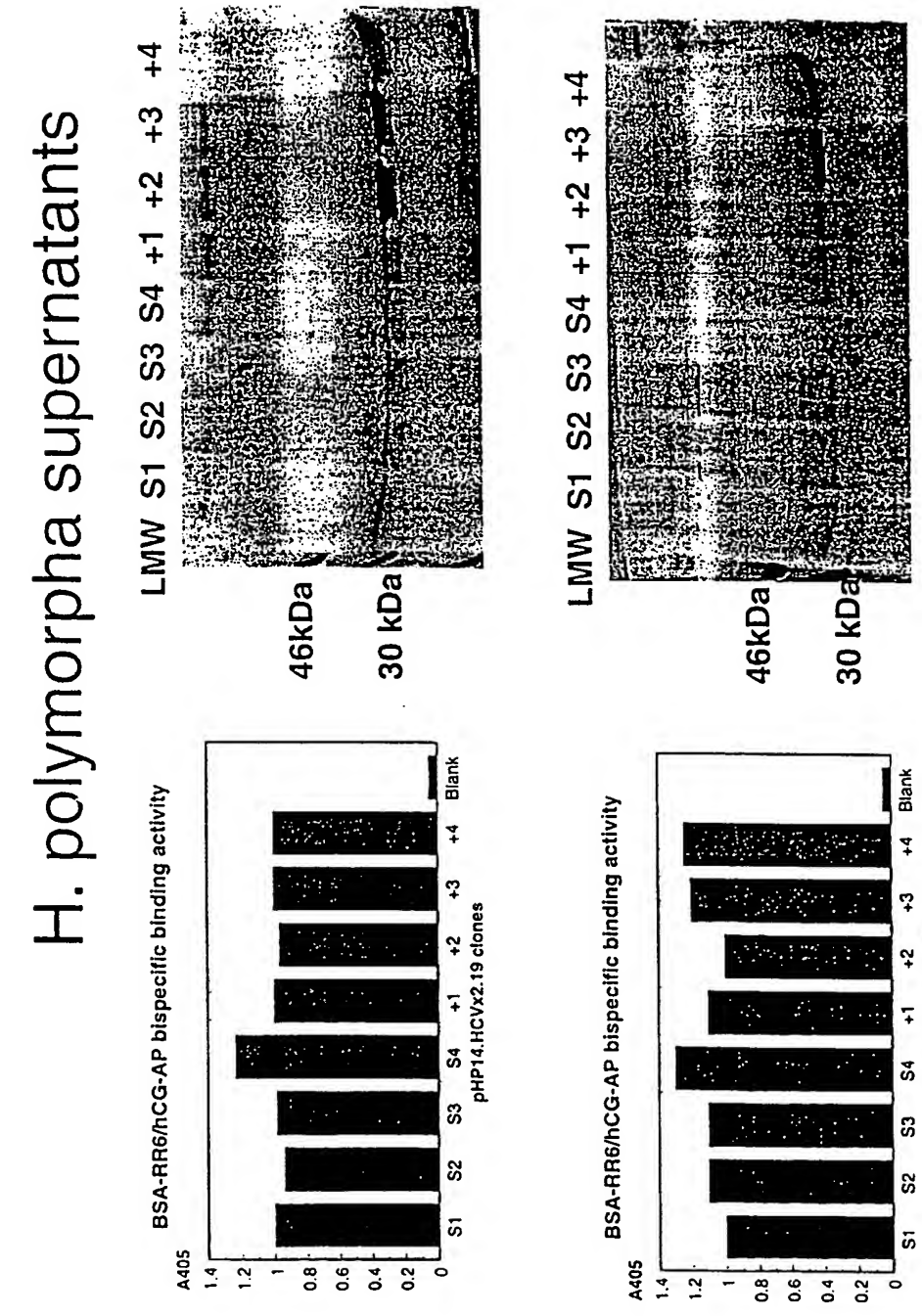


Figure 10 ELISA and SDS-Page analysis of Crude *P. pastoris* supernatants expressing HC-V bihead

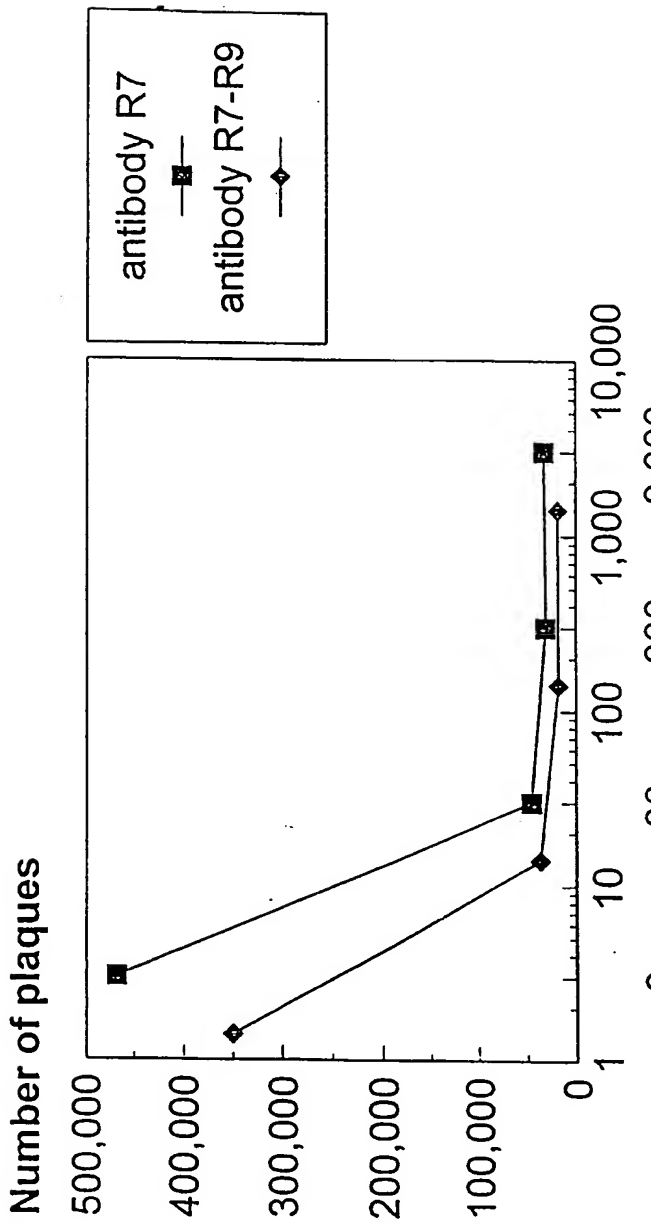


Fig 19 : Effect of monovalent- and bivalent anti-RR6 antibody fragments on the infectivity of RR6 phages

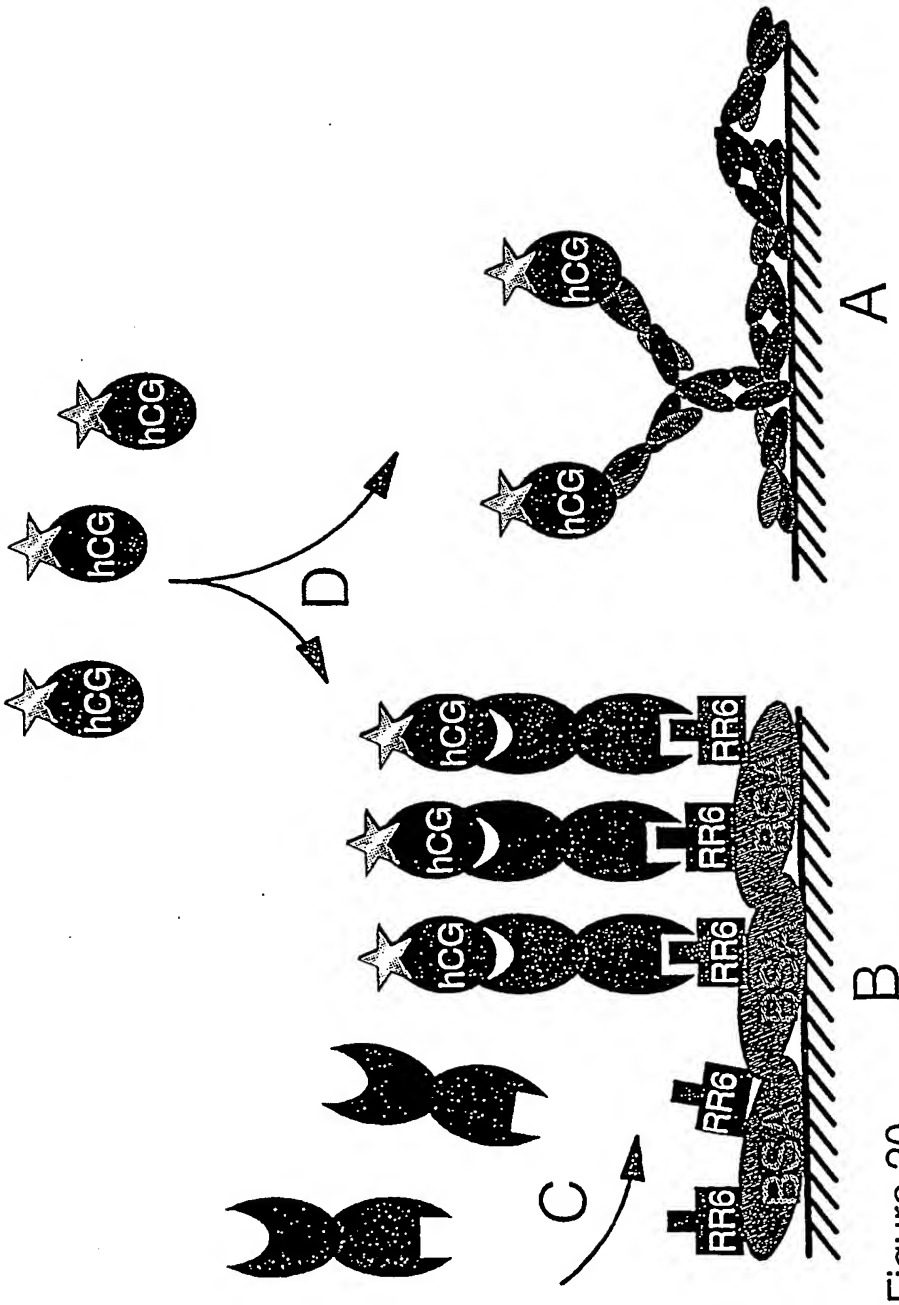


Figure 20.

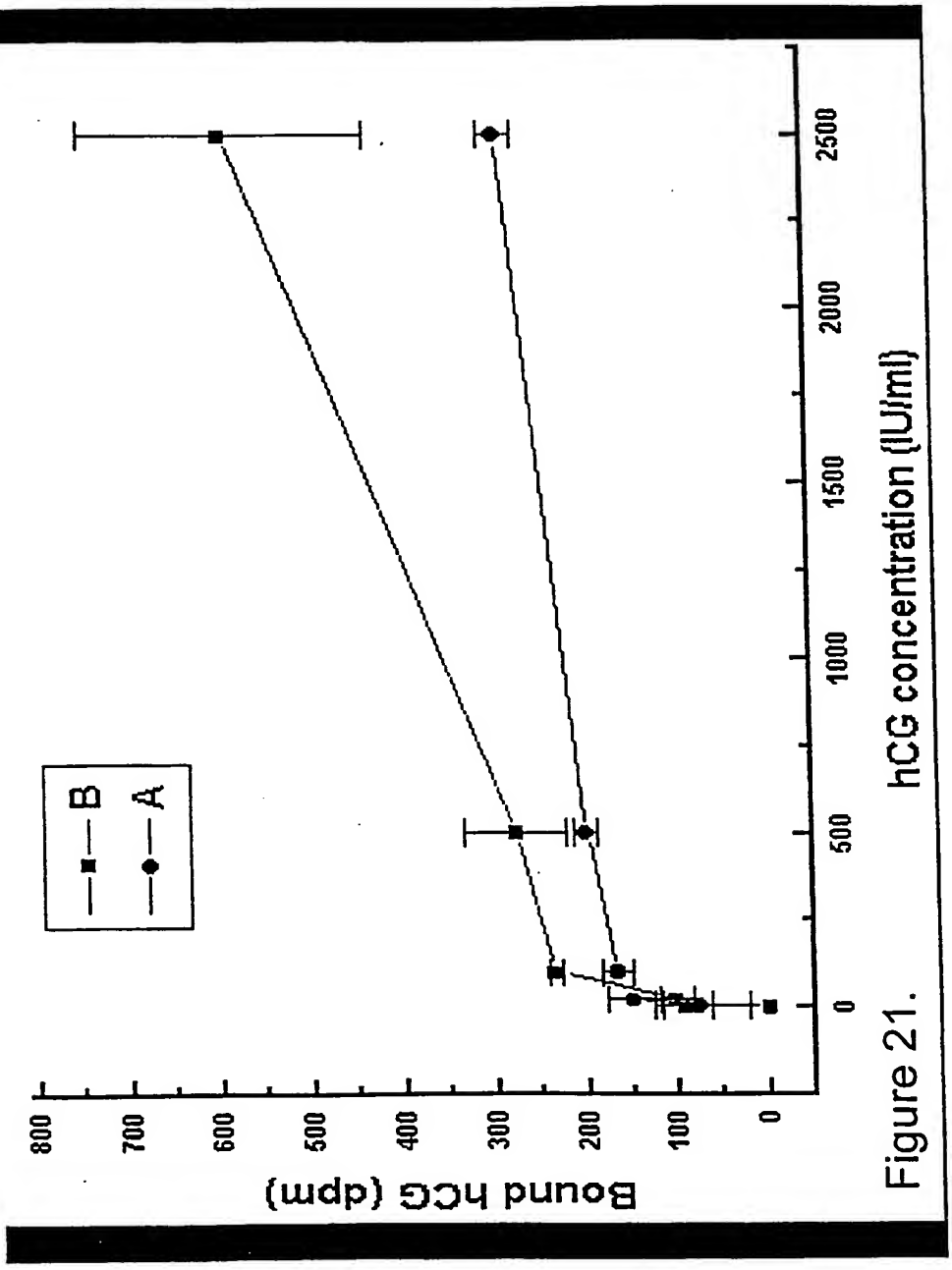

Bound hCG (dpm)
800
700
600
500
400
300
200
100
0
0
500
1000
1500
2000
2500
hCG concentration (IU/ml)
A
B

Figure 21.

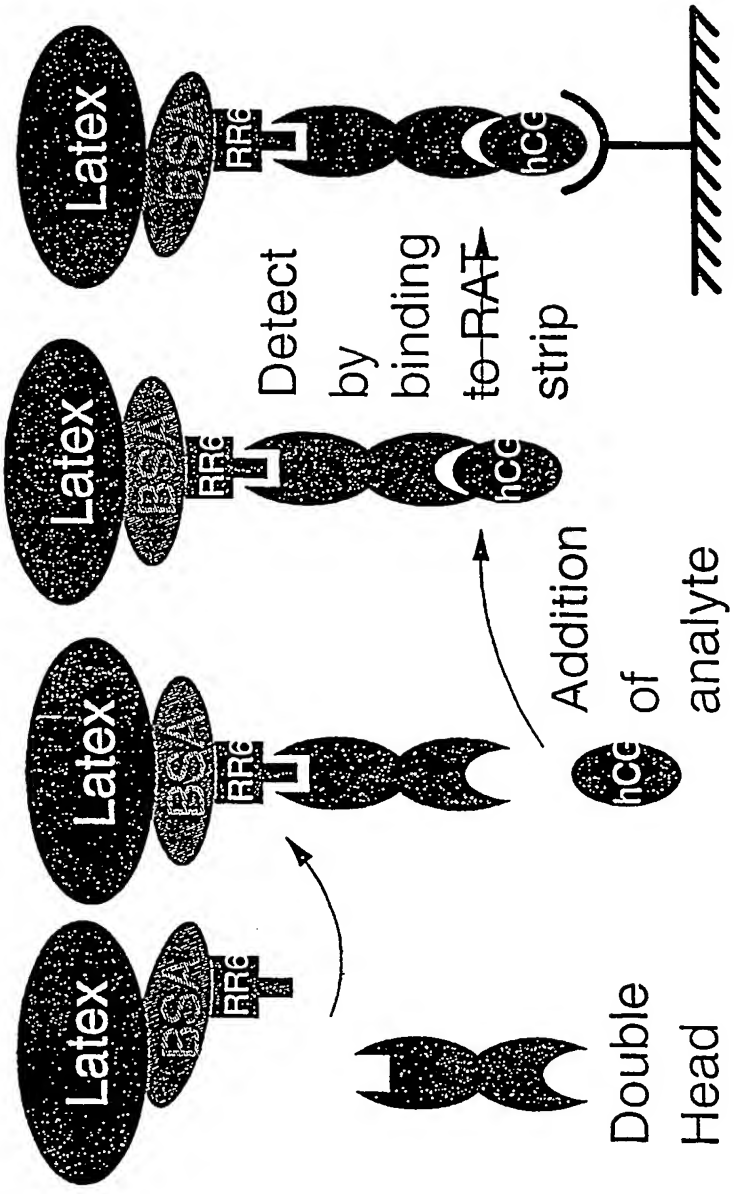

Latex
BSA
RR6
Double
Head
Addition
of
analyte
hCG
Detect
by
binding
to RAT
strip

Figure 22.

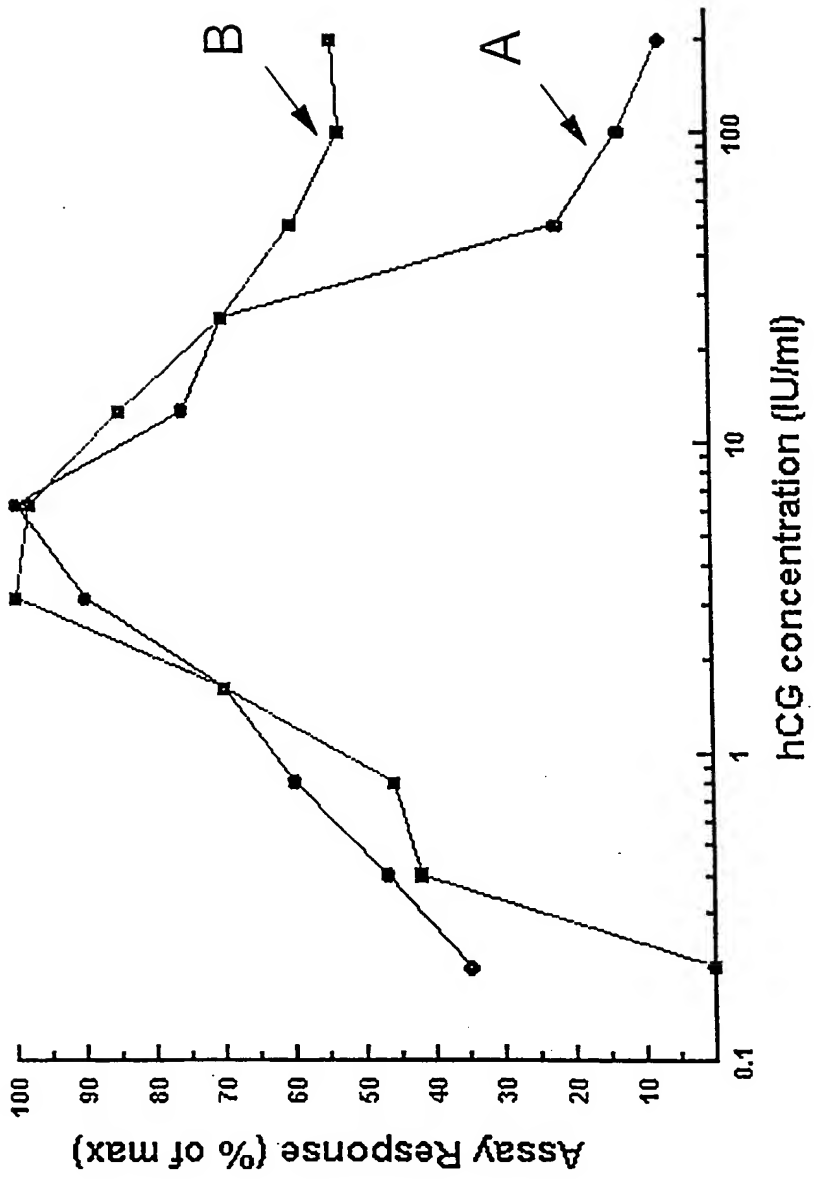

B
A
Assay Response (% of max)
100
90
80
70
60
50
40
30
20
10
0.1
1
10
100
hCG concentration (IU/ml)

Figure 23.

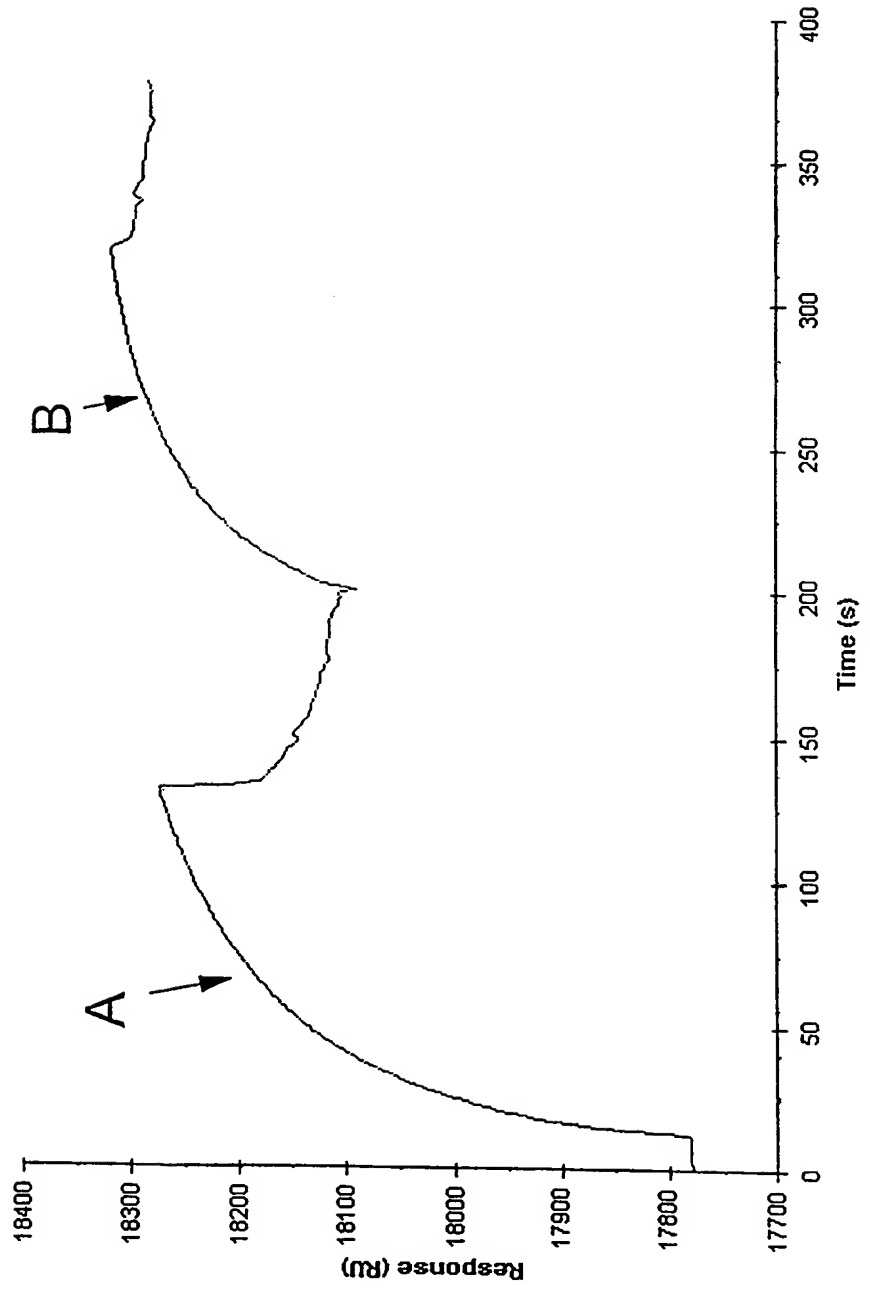

A
B
Response (RU)
18400
18300
18200
18100
18000
17900
17800
17700
0
50
100
150
200
250
300
350
400
Time (s)

Figure 24.

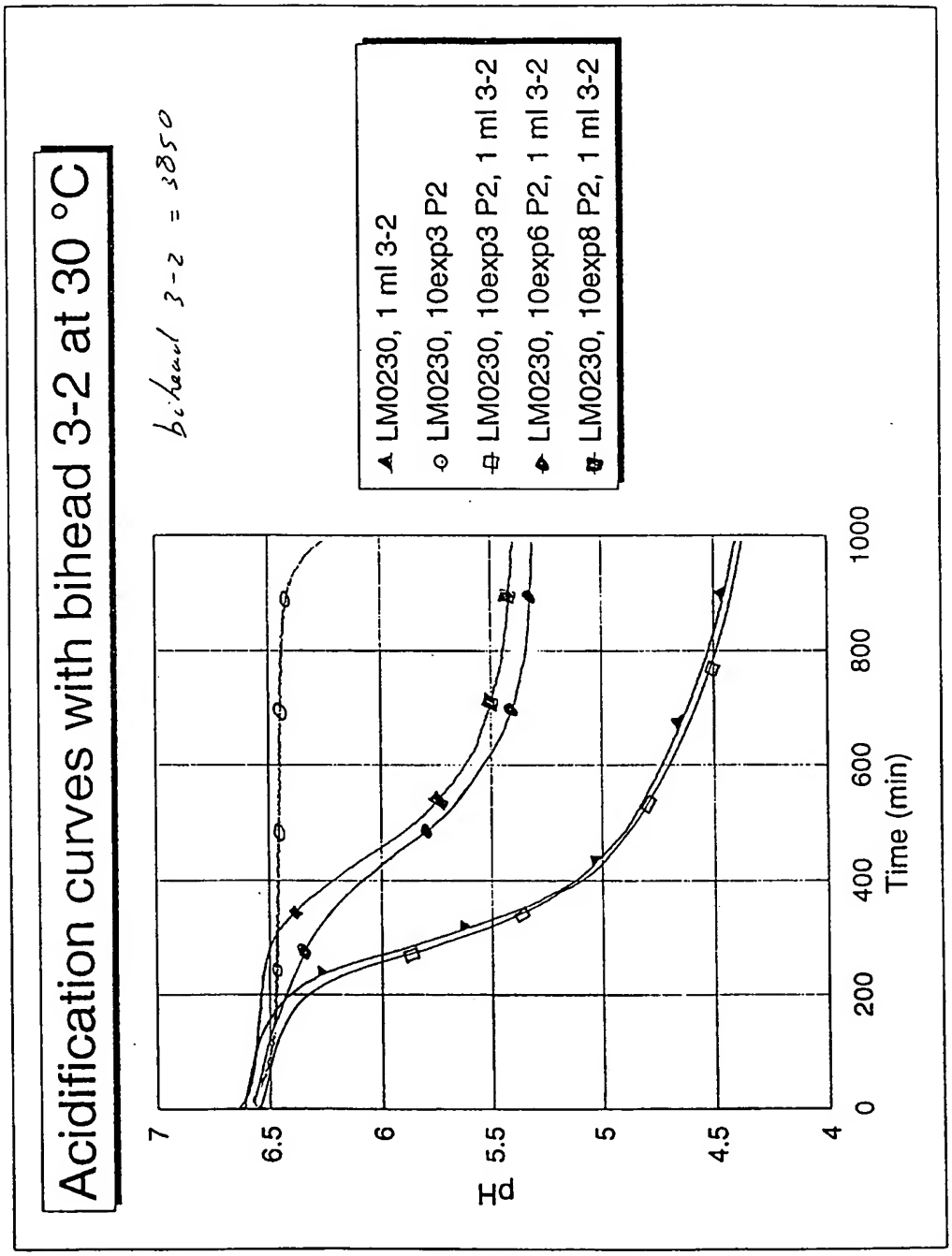

Acidification curves with bihead 3-2 at 30 °C
LM0230, 1 ml 3-2
LM0230, 10exp3 P2
LM0230, 10exp3 P2, 1 ml 3-2
LM0230, 10exp6 P2, 1 ml 3-2
LM0230, 10exp8 P2, 1 ml 3-2
pH
7
6.5
6
5.5
5
4.5
4
0
200
400
600
800
1000
Time (min)

Figure 25

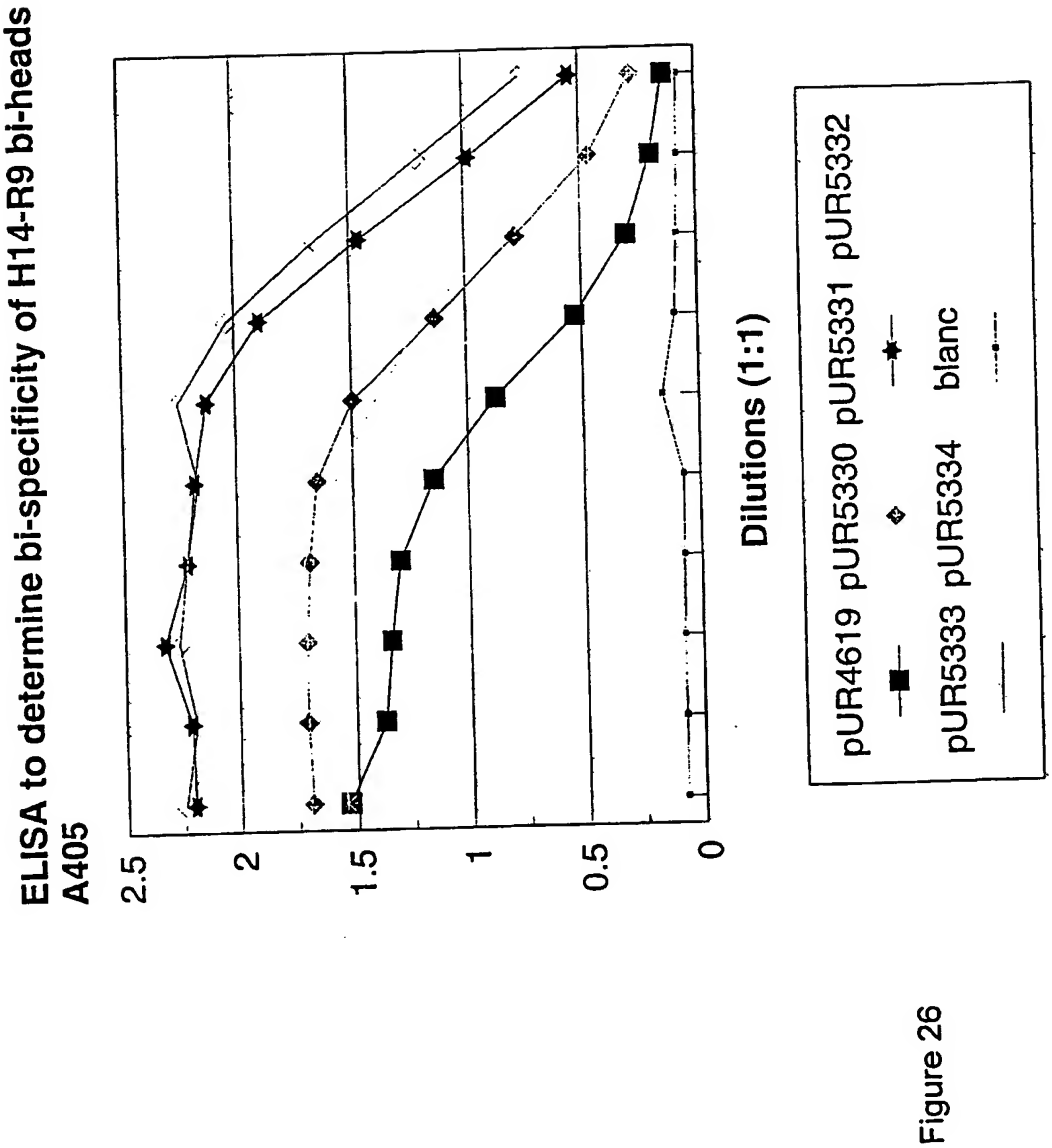

ELISA to determine bi-specificity of H14-R9 bi-heads
A405
2.5
2
1.5
1
0.5
0
Dilutions (1:1)
pUR4619 pUR5330 pUR5331 pUR5332
pUR5333 pUR5334 blanc

Figure 26

Figure 27.

pUR4618 (Bihead H14 - R7)